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14. ABSTRACT Although the importance of microenvironment in prostate cancer is widely recognized, the molecular and cellular processes leading from genetic changes in the prostatic epithelium to the establishment of a tumorigenic microenvironment for prostate cancer is unclear in most contexts. With our finding of NFATc1 being an oncogene and has a potential role in prostate cancer, we proposed to study two main areas (divided into 3 specific aims). First , the detailed study of the tumorigenic microenvironment and the correlation between NFATc1 and prostate cancer status in humans will help facilitate the development of clinically useful biomarkers for both diagnostic and prognostic purposes. Many of the factors we are targeting in the prostate cancer microenvironment are secreted factors that may be present in serum and/or urine at measurable levels, making them suitable for the development of non-invasive clinical tests. Second , the illustration of the main cellular and molecular components in the tumorigenic microenvironment provides new druggable targets aimed at reversing the effects of the alterations in the microenvironment.				
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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Based on our preliminary data revealing a role of NFAT activation in prostate cancer (prostate cancer), we hypothesize that NFATc1 promotes prostate cancer by regulating oncogenic proteins in the prostatic epithelium and by non-cell autonomous effects on other cells through secreted factors. These factors initiate a cascade of reciprocal events between the prostatic epithelium and stroma, leading to the creation of an inflammatory and pro-mitogenic microenvironment for prostate cancer development. Besides testing this hypothesis and to examine the interactions between NFATc1 and known oncogenic factors/tumor suppressors, we will further reveal the key players in the prostate cancer microenvironment and to explore the potential of NFATc1 as a novel biomarker for prostate cancer diagnosis/prognosis. We will take advantage of the cellular precision, genetic manipulability, and on-off inducibility of our murine model to further study the tumorigenic processes initiated by NFATc1 activation in the prostate (Aim 1) as well as the key molecular and cellular components in the NFATc1-induced tumorigenic microenvironment (Aim 2). In Aim 3, we will study the involvement of NFATc1 activation in human prostate cancer and the oncogenic effects of NFATc1 in human prostate cancer cells.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

prostate cancer, microenvironment, oncogene, senescence, NFAT, cytokines,

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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Specific Aims and tasks (specified in proposal)	Timeline (Months)	Site 1 Washington University	Site 2 Tulane University	Actual completion date
Specific Aim 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate				
Major Task 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate				
Subtask 1: Investigate if NFATc1-induced prostate cancer progresses into metastatic prostate cancer	1-24	Drs. Chen, Manda, Tripathi, Ding, Andriole	Dr. You	Completed (Please see details immediately following this table)
Subtask 2: Investigate if NFATc1 promotes the progression of hormone-naive prostate cancer into castration-resistant prostate cancer	1-18	Drs. Chen, Manda, Tripathi, Ding, Andriole	Dr. You	Completed (A portion of these results was described in the previous report and the Oncogene manuscript)
Subtask 3: Investigate if termination of NFATc1 activation halts prostate cancer progression	1-8	Drs. Chen, Manda, Tripathi, Ding,	Dr. You	Completed. 04/2015 (A portion of these results was

		Andriole		described in the previous report and the Oncogene manuscript)
Milestone(s) Achieved: Determine the <i>in vivo</i> role of NFATc1 activation in prostate cancer initiation and progression	24	Drs. Chen, Manda, Tripathi, Ding, Andriole	Dr. You	Some of the results have been included in a manuscript.
Major Task 2: Study the potential synergy between NFAT signaling and Pten/PI3K/Akt in prostate cancer				
Subtask 1: Study if NFATc1 activation overcomes <i>Pten</i> inactivation-induced senescence.	1-6	Drs. Chen Dr. Chen, Manda, Tripathi, Ding, Maher (90 mice will be used)	Dr. You	Completed 05/2015 (A portion of these results was described in the previous report and the Oncogene manuscript)
Subtask 2: investigate if NFATc1 activation promotes prostate cancer bone metastasis in <i>Pten</i> mutants	1-24	Drs. Chen Dr. Chen, Manda, Tripathi, Ding, Maher (90 mice will be used)	Dr. You	Completed (Please see details in sections following this table)
Milestone(s) Achieved: Determine the interactions between NFATc1 and <i>Pten</i> in prostate cancer	24	Drs. Chen, Manda, Tripathi, Ding, Maher	Dr. You	Some of the results have been included in a paper.
Specific Aim 2: Reveal the critical components in NFATc1-induced tumorigenic microenvironment and evaluate the importance of SPP1, a potential NFATc1 target, in NFATc1-induced prostate cancer				
Major Task 3: Study the NFATc1-induced tumorigenic microenvironment and the role of SPP1 in prostate cancer				

Subtask 1: Further analyze the cellular and molecular components in the prostate cancer microenvironment	10-34	Drs. Chen, Tripathi, Manda, Ding (72 mice will be used)	Dr. You	Completed(Please see details in sections following this table)
Subtask 2: Study the role of SPP1, an NFATc1 target, in NFATc1-induced prostate cancer	14-36	Drs. Chen, Tripathi, Manda, Ding (300 mice will be used)	Dr. You	Completed (Please see details in sections following this table)
Milestone(s) Achieved: Provide molecular details to the NFATc1-induced tumorigenic microenvironment and determine the connections between NFATc1 and SPP1	36	Drs. Chen, Tripathi, Manda, Ding	Dr. You	Completed (Please see details in sections following this table)
Specific Aim 3: Investigate NFAT signaling in human prostate cancer specimens and human prostate cancer cell lines				
Major Task 4: Determine if there is a direct connection between NFATc1 expression and human prostate cancer pathogenesis				
Subtask 1: Determine if there is a connection between NFATc1 expression and human prostate cancer grade/stage	1-36	Drs. Chen, Manda, Tripathi, Ruzinova, Hsi, Ding, Maher, Andriole (275 human prostate cancer specimens)		Completed (Please see details in sections following this table)
Subtask 2: Investigate the oncogenic effects of NFAT signaling in human prostate cancer cell lines	16-32	Drs. Chen, Manda, Tripathi, Ding, Maher	Dr. You	Completed (Please see details in sections following this table.)
Milestone(s) Achieved: Determine if NFATc1 can be a biomarker for	36	Drs. Chen, Manda,		Completed (Please see details

prostate cancer progression in human and further understand the effect of NFATc1 activation in human prostate cancer cells		Tripathi, Ruzinova, Hsi, Ding, Maher, Andriole		in sections following this table)
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What was accomplished under these goals?

1) Major activities

Major Task 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate

We have completed the study of the effects of antigen deprivation in mice with NFAT activation in the prostate.

We have completed the study if termination of NFATc1 activation halts prostate cancer progression in the NFATc1-induced murine prostate cancer model.

We have so far not found any evidence of metastasis of the NFATc1-induced prostate cancer in mice.

Major Task 2: Study the potential synergy between NFAT signaling and Pten/PI3K/Akt in prostate cancer

We have demonstrated the synergy between NFAT signaling and Pten/PI3K/Akt signaling in prostate cancer.

We have shown that NFATc1 has anti-senescence effects and such effects overcome Pten inactivation-associated cellular senescence.

Major Task 3: Study the NFATc1-induced tumorigenic microenvironment and the role of SPP1 in prostate cancer

We have further analyzed the cellular and molecular components in the prostate cancer microenvironment.

We have performed RNA-Seq to further study the changes in molecular pathways when NFATc1 is activated in PCa.

Major Task 4: Determine if there is a direct connection between NFATc1 expression and human prostate cancer pathogenesis

We have studied various human PCa specimens to determine the connection between NFATc1 expression and human prostate cancer progression.

We have investigated the oncogenic effects of NFAT signaling in human prostate cancer cell lines. These studies have provided useful information about the molecular and cellular mechanism of NFATc1-driven oncogenesis in the prostate and other organs.

2) Specific objectives

Our main objectives are:

Aim 1: Investigate the tumorigenic processes initiated by NFATc1 activation in the prostate.

Aim 2: Reveal the critical components in NFATc1-induced tumorigenic microenvironment and evaluate the importance of SPP1, a potential NFATc1 target, in NFATc1-induced prostate cancer.

Aim 3: Explore the potential of NFATc1 as a novel diagnostic/prognostic marker and study the role of NFATc1 in human prostate cancer cell lines.

3) Significant results

Major Task 1: Investigate the tumorigenic processes initiated by NFATc1 activation in prostate

1.1: Study the effects of castration in mice with NFAT activation in prostate:

Androgens are critical both for development and function of the prostate gland and for the survival and proliferation of the epithelial cells.¹ In order to determine if NFATc1-induced PCa would respond to hormone deprivation therapy, such as castration, we analyzed prostates from 18-week-old mutant mice with NFATc1 activation since weaning and were either castrated (by surgically removing both testicles) or mock-castrated at 14 weeks of age. Unlike tumor allografts grown subcutaneously in the nude mice, the size of the tumors in the prostate is much harder to measure technically. We thus only compared

the results between castrated and mock-operated mice at the end point (18 weeks of age). PCa samples from castrated and mock-castrated mutants are similar in tumor size and histopathological features (Fig. 1).

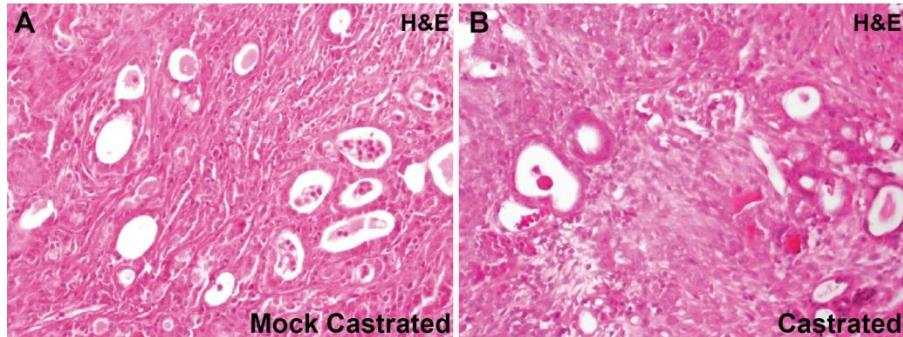


Figure 1: Castration did not prevent NFATc1-induced PCa formation.

Representative images of H&E stained sections of tumors from mock-castrated and castrated mice showing PCa.

However, the distinct nuclear AR staining seen in the prostate of non-castrated mice was replaced by a weaker and more diffused expression pattern in castrated mice. The reduction but not absence of AR staining is consistent with observations in other castration experiments,^{2,3} potentially due to androgen independent AR signaling and other reasons. These results indicate that castration had reduced AR signaling in prostatic cells (Fig. 2A-B).

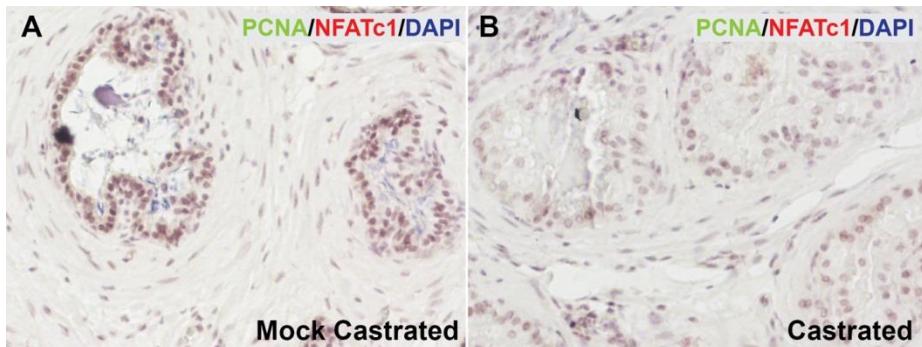


Figure 2: Castration causes reduced AR signals in prostatic epithelia.

Prominent nuclear AR is present in mock-castrated mice (A). AR signal is weaker and more diffuse in the sample from the castrated mutants (B).

Normal adult prostates generally did not show significant number of PCNA+ proliferating cells. They also lack NFATc1+ cells (Fig. 3A). On the contrary, tumors from mock-castrated mice had significant number of PCNA⁺ proliferating cells. Tumors from the castrated mice also have high levels of PCNA+ cell, indicating that castration did not prevent cell proliferation and NFATc1-induced PCa (Fig. 3B-C).

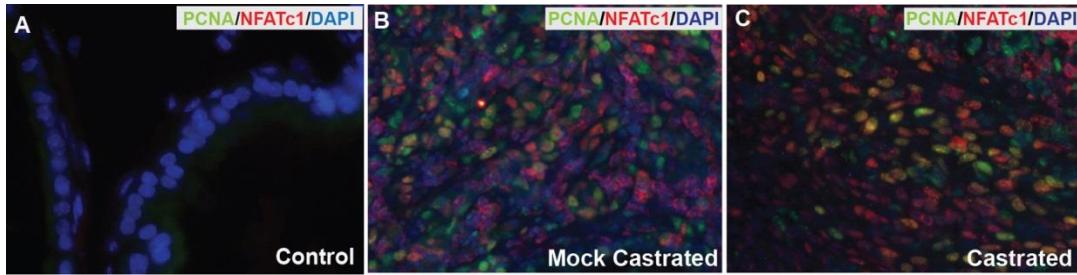


Figure 3: Castration did not stop prostatic epithelial cell proliferation in NFATc1-induced PCa.

A: Control adult prostates are generally void of proliferating cells and NFATc1 staining. Proliferating cells (revealed by green PCNA staining) are present in PCa of the mock-castrated mutants (B). PCa from castrated mutants continues to have significant numbers of proliferating cells (C), despite that the testes of these mice were surgically removed.

1.2: Study if termination of NFATc1 activation halts prostate cancer progression in the murine prostate cancer model

To directly test the essential role of NFATc1 activation in tumorigenesis, we studied the ability of the cells with NFATc1 activation to initiate tumorigenesis in nude mice. We derived tumor cells from NFATc1-induced murine PCa and showed that about 70% of these cells expressed NFATc1 and the HA (human influenza hemagglutinin) tag fused to the C-terminus of NFATc1 (Fig. 4A). These cells were injected to the rear flanks of the nude mice. Since these cells were cultured for multiple passages before the injection, no suspension cells (especially lymphocytes) were included. Tumor growth was detected as early as 4 weeks after the injection in the Dox-treated (with NFATc1 activation), but not in the untreated (without NFATc1 activation), recipient mice (Fig. 4B-C).



Figure 4: Allografts of NFATc1-induced tumors showed dependency on NFATc1 for tumor progression and survival.

Cells from NFATc1-induced PCa samples were isolated and cultured. Most of the cultured cells expressed NFATc1 and the HA tag (A). Cultured tumor cells were injected subcutaneously into the lower flanks of nude mice. 100% of the Dox-treated recipients developed tumors by 4 weeks, whereas none of the untreated mice did (B-C).

To

further test the dependency of tumor growth and progression on NFATc1 activation, we stopped Dox

treatment in a subgroup of these mice. Existing tumors started to shrink within days after Dox withdrawal (Fig. 5). This trend was reversed when NFATc1 activation was restored with Dox treatment (Fig. 5), indicating a continuous dependency of the PCa on NFATc1 activation, similar to that seen in cases of oncogene addiction.⁴⁻⁶

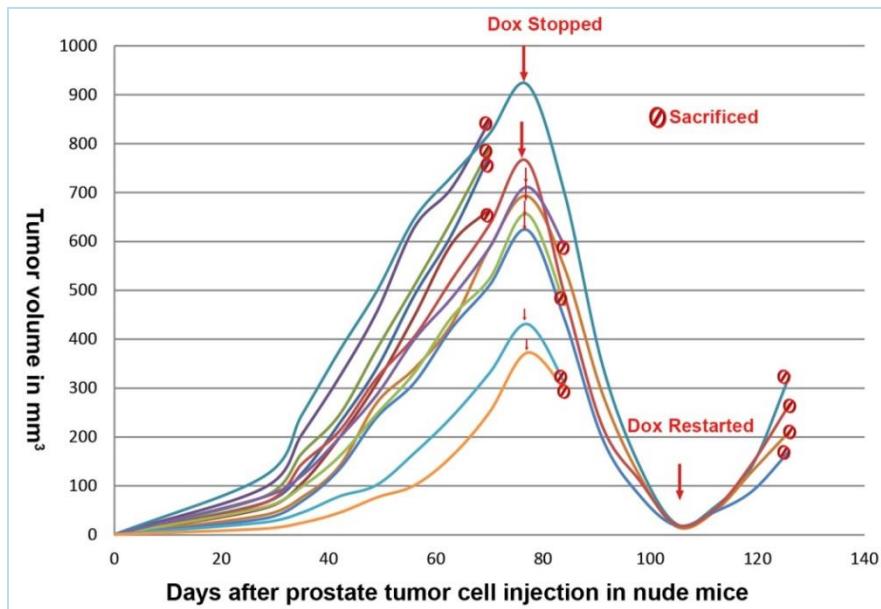


Figure 5: Allografts of NFATc1-induced tumors showed continuous dependency on Dox-induced NFATc1 Activity for tumor progression.

Termination of Dox treatment resulted in significant decrease in tumor size. Such decrease was reverted if Dox treatment was restarted.

Histopathological analyses of tumors revealed that these allografts contained carcinoma with a more solid growth pattern but showed cytological features similar to those seen in original tumors (Fig. 6A), including the presence of a large number of NFATc1⁺/E-Cad⁺ cells (Fig. 6B) and STAT3 activation in both NFATc1⁺ and NFATc1⁻ cells that intermingled within the tumor proper (Fig. 6C). Inflammatory cytokines, such as IL6, are similarly upregulated in the grafts (Fig. 6D). Since the control mice in this experiment did not have any tumor growth (as expected), it would not be informative to compare the tumor allograft to the host without tumor. For the purpose of comparison, the normal adult prostate has essentially no detectable levels of proliferating cells, and no detectable level of expression of NFATc1, pSTAT3, and IL6 (Fig. 6E-L).

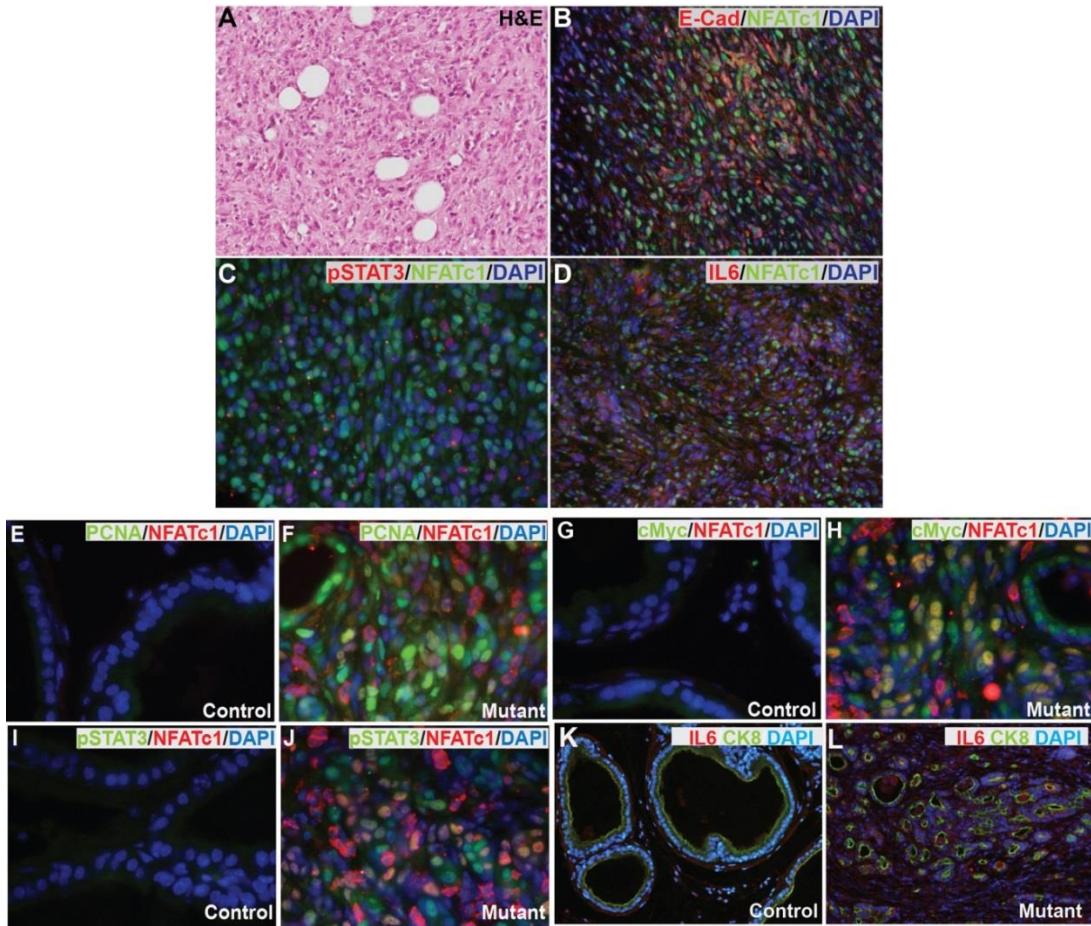


Figure 6: Allografts of NFATc1-induced tumors showed similar promitogenic and inflammatory microenvironment.

Representative images of H&E stained nude mice allograft (A). The allograft tumors predominantly consist of NFATc1⁺ cells expressing E-Cad (B). Similar to the original tumor, extensive pSTAT3 (C) and IL6 (D) expression was observed in the allograft tumors. E-L: Comparison of expression of selected markers between control and mutants (with NFATc1 activation in prostatic epithelia). Since the controls in the tumor grafting experiments are the ones without tumor formation, we have thus provided here images from our immunofluorescence staining showing the absence of NFATc1, pSTAT3, and IL6 in normal mouse prostates and the detection of these proteins in the NFATc1-induced PCa.

1.3: Investigate if NFATc1-induced prostate cancer progresses into metastatic prostate cancer

Several studies have shown that NFATc1 is associated with the progression of multiple types of cancers.⁷⁻²³ To determine whether NFATc1-induced prostate cancer in mice can metastasize, we sacrificed and analyzed the lymph nodes, lung, liver and bones (mainly tibia, femur and spinal vertebrae) of mice with induced NFATc1 activation in the prostatic epithelium for various length of time, from 14 weeks to about 6 months. Due to the overall tumor burden and other confounding problems in

these mice, only 2 mice lived past 6 months after NFATc1 activation. Figure 1 has representative data from one of such mutant mice.

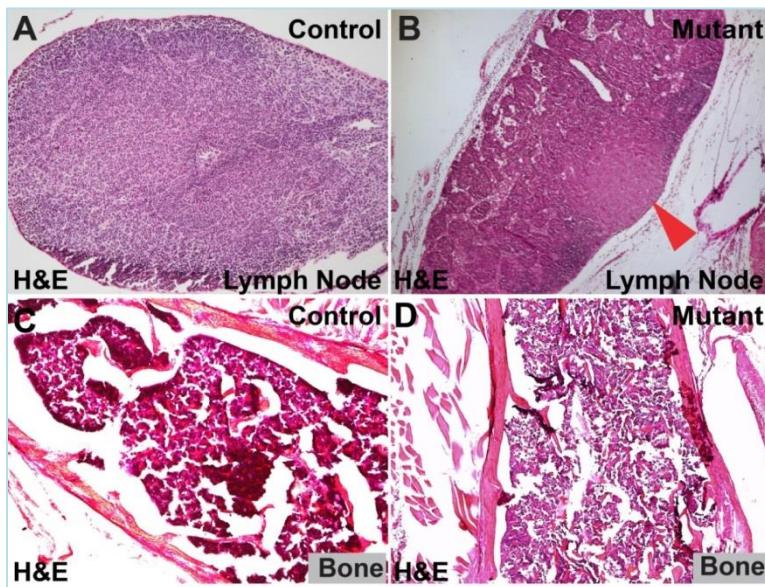


Figure 7. No clear evidence of metastasis in mice with NFATc1-induced prostate cancer has been found.

We have found some unexplained and unusual cells and cell masses in the lymph nodes occasionally (Figure 7A-B), but we have not found any clear evidence of metastasis in the bones (Figure 1C-D) or other organs (data not shown). It is still unclear what type of cells made up the unusual mass in some of the mutant lymph nodes. Morphologically, they do not appear to resemble prostate cancer cells. Their identity and significance are still under investigation. It is possible that these are results of immune responses rather than metastasis. Although we are still looking at more samples with longer duration of NFATc1 activation, we have shifted our attention to mutants with both NFATc1 activation and PTEN inactivation to test the hypothesis that additional genetic mutations may promote the NFATc1-induced prostate cancer to progress further, including metastasis. Please see section 2.3.

Major Task 2: Study the potential synergy between NFAT signaling and Pten/PI3K/Akt in prostate cancer

2.1: Study the synergy between NFAT signaling and Pten/PI3K/Akt signaling in prostate cancer.

We found that NFATc1 activation synergizes with the PI3K-AKT pathway to promote PCa progression. Pten is one of the most frequently mutated tumor suppressors in PCa.^{24, 25} To understand if and how the NFAT and PI3K-AKT pathways interact in PCa, we generated mice with both Pten deficiency and

NFATc1 activation in prostatic epithelia. At 10 weeks of age, most PCre/+;Pten^{f/f} mice with only PTEN deficiency in the prostate epithelium showed enlarged anterior prostates, whereas control and PCre/+;RT/+;TN/+ mice with only NFATc1 activation starting from P21 in prostatic epithelium had no visible tumors. Interestingly, all double mutants (PCre/+;RT/+;TN/+;Pten^{f/f}) with both PTEN deficiency and NFATc1 activation developed significantly larger tumors in all prostate lobes when compared to mice of the same age with either Pten deficiency or NFATc1 activation alone (Fig. 8A-D). The average prostate weight in double mutants (6026.24 ± 1946.85 mg) was increased 17.41-fold when compared to the controls (346.85 ± 36.66 mg), 15.45-fold when compared to mice with NFAT activation alone (390.28 ± 73.16 mg), 7.35-fold when compared to Pten null mice (819.14 ± 139.4 mg, Fig. 8E).

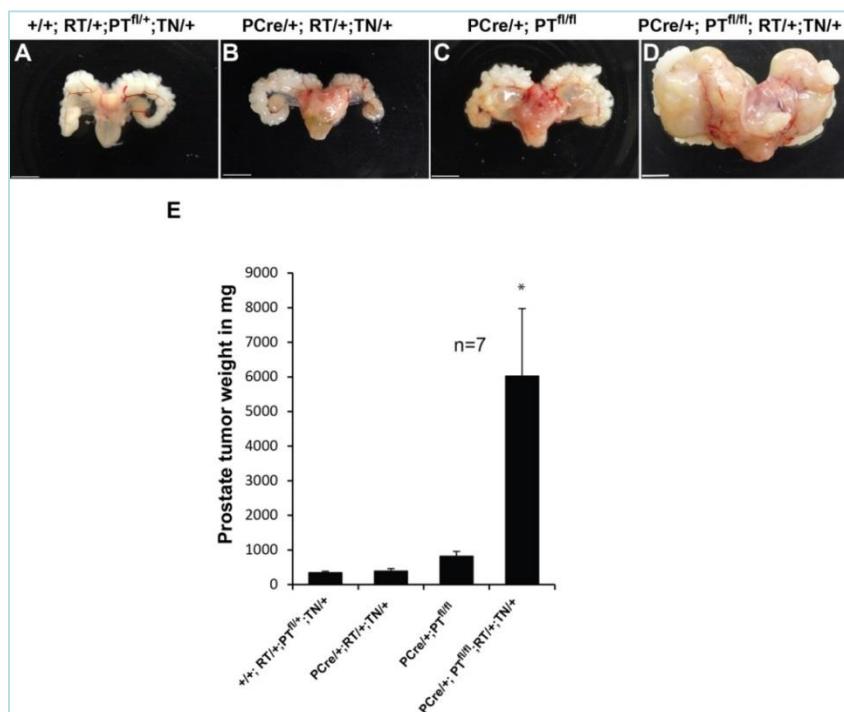


Figure 8: NFATc1 and PI3K-Akt signaling pathway synergize to drive accelerated tumor formation.

Representative images of tumors with PCre/+;RT/+;TN/+;Pten^{f/f} double mutants showing significantly enlarged tumors compared to control (no NFATc1 activation or Pten deletion), PCre/+;RT/+;TN/+ (NFATc1 activation alone), and PCre/+;Pten^{f/f} (Pten deletion alone) groups (A-D). Average whole prostate weight of the PCre/+;RT/+;TN/+;Pten^{f/f} mice is drastically higher than those of the +/+, RT/+, TN/+; Pten^{f/f} mice (* $p < 0.05$, N=7) PCre/+;RT/+;TN/+ mice (** $p < 0.05$, N=7), and PCre/+;Pten^{f/f} mice (** $p < 0.05$, N=7). (E). All data are presented as mean \pm s.d. Two-tailed t-tests were performed for comparison between groups.

Histopathological analyses revealed that Pten null mice and mice with NFATc1 activation alone had PIN at this time, whereas double mutants already had poorly differentiated prostatic adenocarcinoma (Fig. 9A-D). While levels of pAKT were low in prostates from controls and mice with only NFATc1 activation, increased expression of pAKT was apparent in PCre/+;Pten^{f/f} and PCre/+;RT/+;TN/+;Pten^{f/f}

samples, indicating that the PI3K-AKT pathway was activated in prostates with PTEN loss (Fig. 9E-H). SMA staining revealed intact myofibroblast layers in the prostates from single mutants but widespread disintegration of the SMA layer in double mutants, consistent with invasion of the epithelial cells into the stroma (Fig. 9I-L). These findings reveal that NFATc1 activation synergizes with PTEN-AKT pathway for PCa initiation and progression.

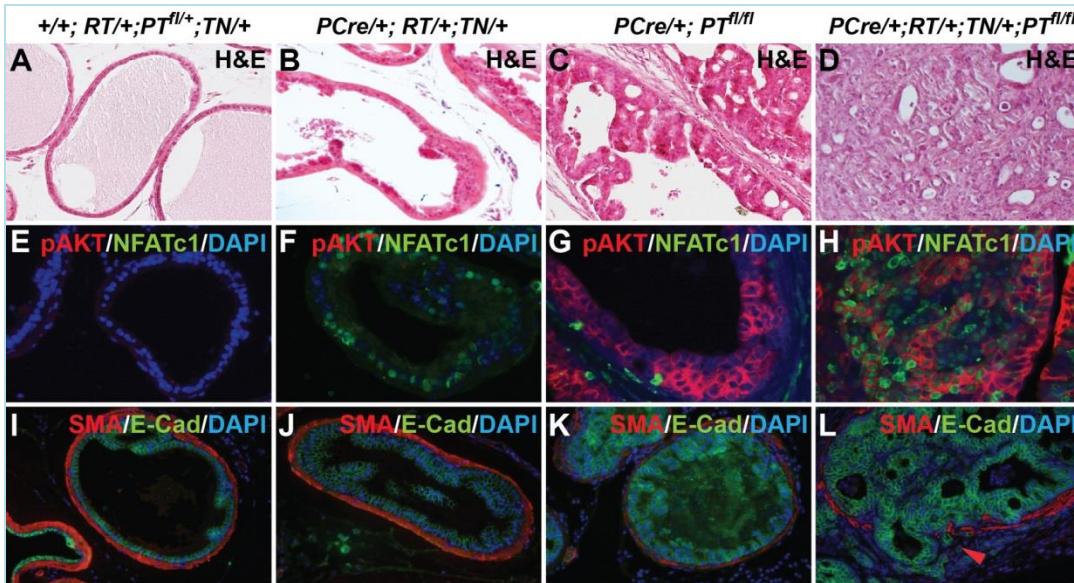


Figure 9: NFATc1 activation and the inactivation of Pten have synergistic effects on PCa.

H&E staining of prostates at 10 weeks of age reveals normal glands in controls, PIN in *PCre/+;RT/+;TN/+* mice, morphologically more advanced PIN in *PCre/+;Pten^{f/f}* mice, and advanced PCa in *PCre/+;RT/+;TN/+;Pten^{f/f}* double mutant mice (A-D). Deletion of Pten results in activation of AKT in *PCre/+;Pten^{f/f}* and *PCre/+;RT/+;TN/+;Pten^{f/f}* mutant mice whereas no significant levels of pAKT were detected in control and NFATc1 activation only groups (E-H). Discontinuation of the SMA⁺ fibromuscular layer and invasion of the E-Cad⁺ cells into the stroma (arrowhead in L) can be seen in the mutants (I-L).

2.2: Study if NFATc1 has anti-senescence effects and if such effects overcome Pten inactivation-associated cellular senescence.

We found that NFATc1 activation overcomes PTEN-loss-induced cellular senescence through down regulation of cell cycle inhibitors. Senescence plays a tumor-suppressive role in PTEN-deficient cells, explaining the long tumor latency in murine models with PTEN-deficient prostate.²⁵ It has been shown that senescence plays a tumor-suppressive role in PTEN-deficient cells, explaining the long tumor latency in murine models with PTEN-deficient prostate.²⁵ The earlier onset and faster progression of PCa in double mutants suggest that NFATc1 activation may allow the tumor cells to avoid the PTEN-loss-induced cellular senescence, resulting in accelerated tumor growth. Therefore, we examined markers of proliferation and senescence in prostates from (*PCre/+;Pten^{f/f}*), (*PCre/+;RT/+;TN/+*), and

(PCre/+;RT/+;TN/+; Pten^{f/f}) mice. The PCre/+;RT/+;TN/+;Pten^{f/f} tumors had significantly higher levels of proliferation ($68.87 \pm 18.37\%$) than the PCre/+;Pten^{f/f} ($24.16 \pm 6.76\%$), and PCre/+;RT/+;TN/+ ($33.46 \pm 3.72\%$) tumors, as assessed by PCNA staining (Fig. 10).

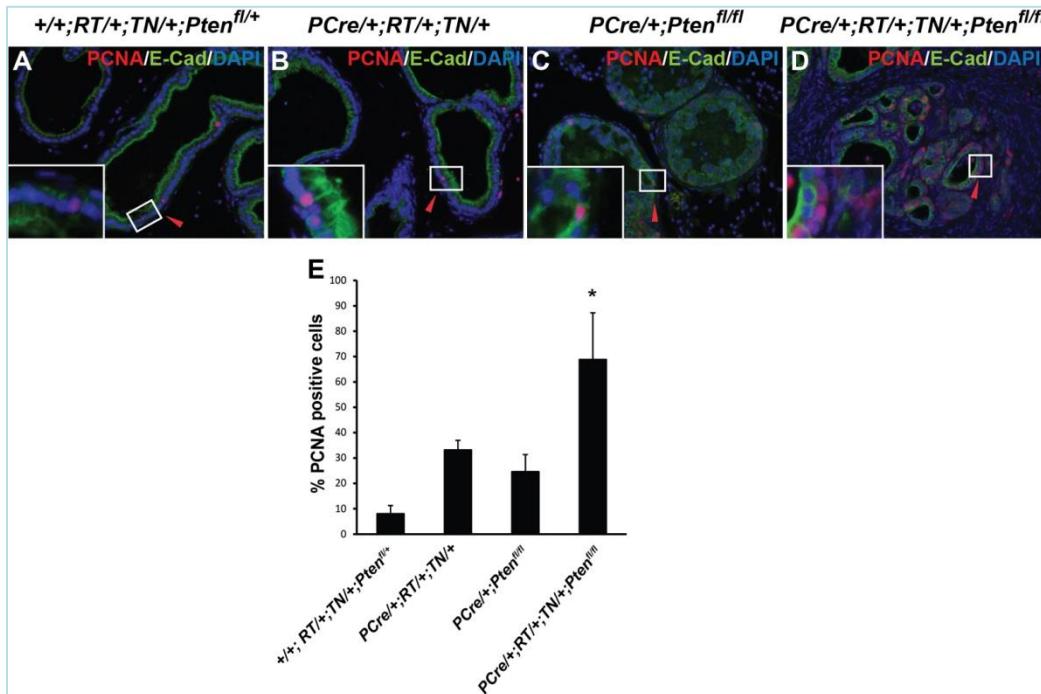
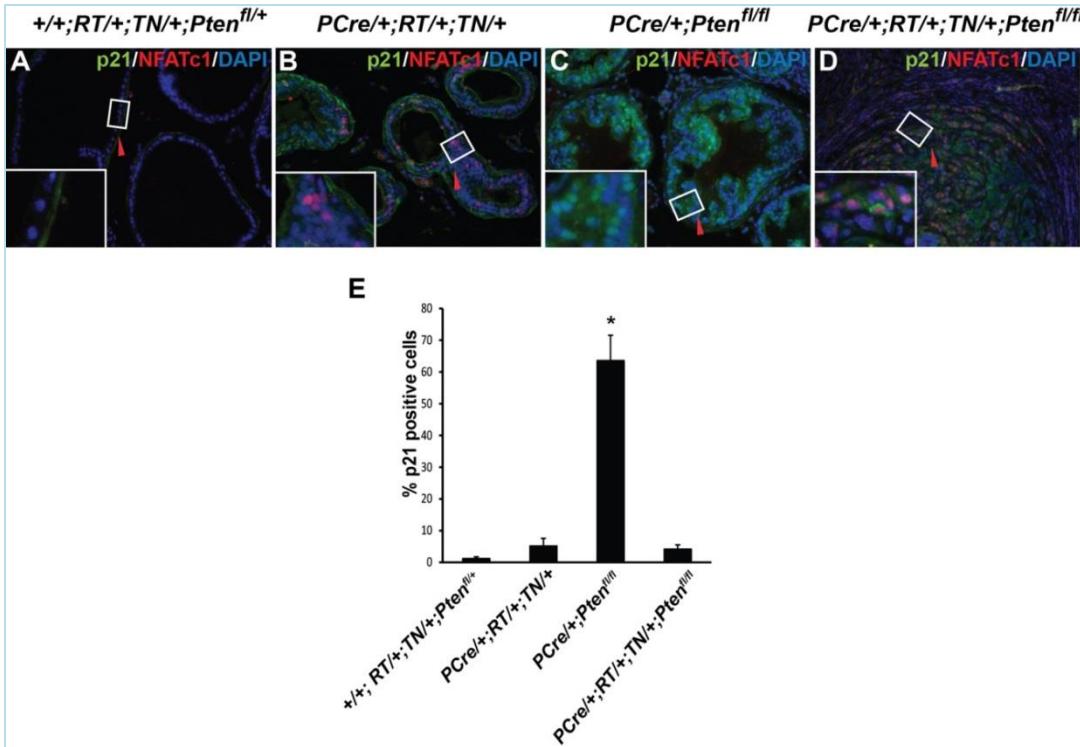


Figure 10: NFAT activation and PTEN inactivation have synergistic effects on cell proliferation in PCa.

PCre/+;RT/+;TN/+;Pten^{f/f} mutant prostates have a much larger number of proliferating E-Cad⁺ cells when compared to control, PCre/+;RT/+;TN/+, and PCre/+;Pten^{f/f} mice (A-D). The insets are higher magnification images of the white rectangles indicated by arrows. Quantification of PCNA staining of 10-week-old prostates is shown in E Asterisk indicates statistical significance between PCre/+;RT/+;TN/+;Pten^{f/f} double mutants and PCre/+;Pten^{f/f} single mutants (* $p < 0.05$, N=5).

Furthermore, there was a marked decrease in the expression of the senescence marker p21 in PCre/+;RT/+;TN/+;Pten^{f/f} samples when compared with the PCre/+;Pten^{f/f} mice (Fig. 11A-D, arrowhead and inset). p21 staining was predominantly nuclear in PCre/+;Pten^{f/f} prostates ($63.6 \pm 7.95\%$). In contrast, nuclear p21 expression was absent in PCre/+;RT/+;TN/+;Pten^{f/f} ($4.2 \pm 1.30\%$) prostates, where cytoplasmic p21 was occasionally observed (Fig. 11E). While nuclear p21 is considered as tumor suppressors, cytoplasmic p21 may have anti-apoptotic roles and enhance cell survival.^{26, 27}



To further confirm that NFATc1 activation overcomes PTEN-loss-induced cellular senescence, we stained for senescence-associated β -galactosidase (SA- β -gal) activity in the prostates. Control and *PCre/+;RT/+;TN/+* prostates showed very few senescent cells, 1% and $6.66 \pm 0.5\%$, respectively. In contrast, $65.6 \pm 8.7\%$ cells within the *PCre/+;Pten^{f/f}* prostates were SA- β -gal⁺. Such SA- β -gal⁺ cells in the *PCre/+;RT/+;TN/+;Pten^{f/f}* prostates were dramatically reduced to $5.8 \pm 1.3\%$ (Fig. 12), supporting the hypotheses that NFATc1 overcomes Pten-induced cellular senescence by down regulating cell cycle inhibitors

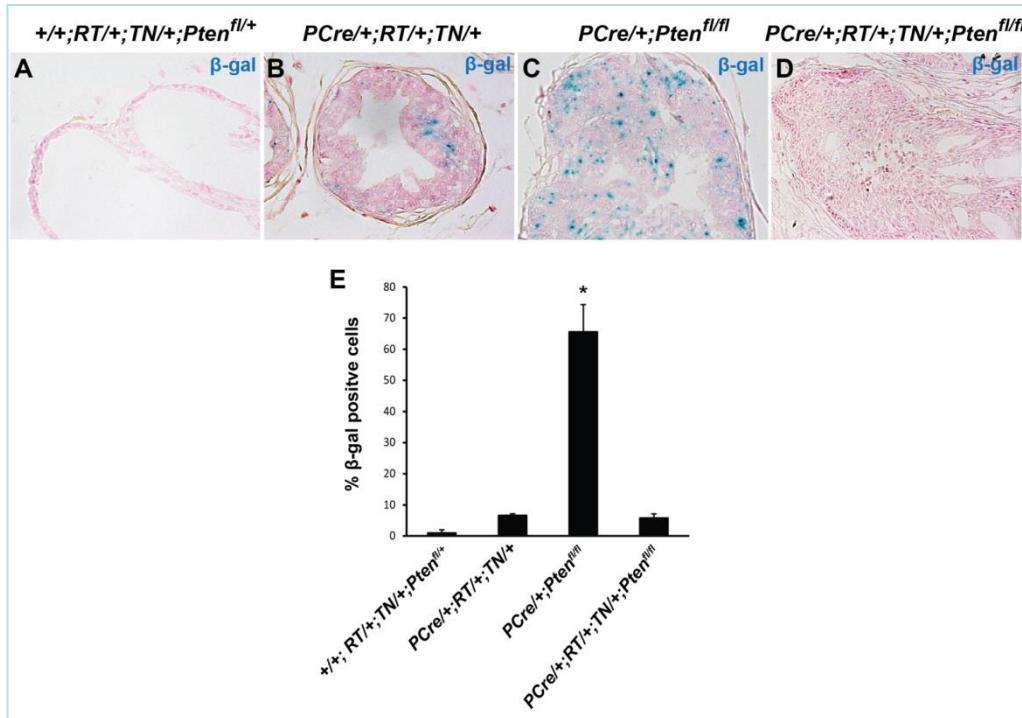


Figure 12: NFAT Activation overcomes PTEN-loss-induced cellular senescence in PCa.

A-D: Senescence-associated β-gal staining of prostates from 10-week-old mice. Quantification of SA-β-gal⁺ cells in 10-week-old prostates (**E**) is consistent with the p21 data. Prostates from control and *PCre+/+;RT+/+;TN+/+* mice had few SA-β-gal⁺ senescent cells. Prostates of the *PCre+/+;Pten^{fl/fl}* mice contain a large number of SA-β-gal⁺ senescent cells, when compared to prostates of the *PCre+/+;RT+/+;TN+/+;Pten^{fl/fl}* double mutants that had significantly fewer SA-β-gal⁺ senescent cells (**p* <0.05, *N*=5). All data are presented as mean ± s.d. Two-tailed t-tests were performed for comparison between groups. Asterisk indicates statistical significance between the *PCre+/+;Pten^{fl/fl}* single mutants and the *PCre+/+;RT+/+;TN+/+;Pten^{fl/fl}* double mutants.

2:3: Investigate if NFATc1 activation promotes prostate cancer bone metastasis in Pten mutants

In our earlier aims, we have shown that NFATc1 activation interacts synergistically with the PI3K-PTEN-AKT pathway in driving the progression of the prostate cancer in mice by overcoming PTEN loss-induced senescence. We next tried to determine if NFATc1-induced prostate cancers with the additional PTEN inactivation can metastasize to other organs. Since prostate cancers in the double mutants grow very aggressively and cause severe morbidity and death in the mice bearing these cancers, we have so far only managed to analyze double mutant mice with NFATc1 activation for about 7 weeks. We harvested and analyzed the lymph nodes, lung, liver and bones (mainly tibia, femur and spinal vertebrae) from the mutant and control mice treated with Dox for up to 7 weeks. We used H&E and immunofluorescence staining to determine if any metastases occurred. Briefly, we have not found clear evidence of metastasis of the NFATc1-induced prostate cancer in bone and other organs in these relatively young mice (Figure 13). There were occasional unusual findings of NFATc1+ cells in the

mutants, such as in the lung section in Figure 13B. However, since these cells are not E-Cad positive, we do not consider them to be metastatic prostate cancer cells. While local invasions occurred, we have found no concrete evidence to support the hypothesis that NFATc1-induced prostate cancers can metastasize to other organs.

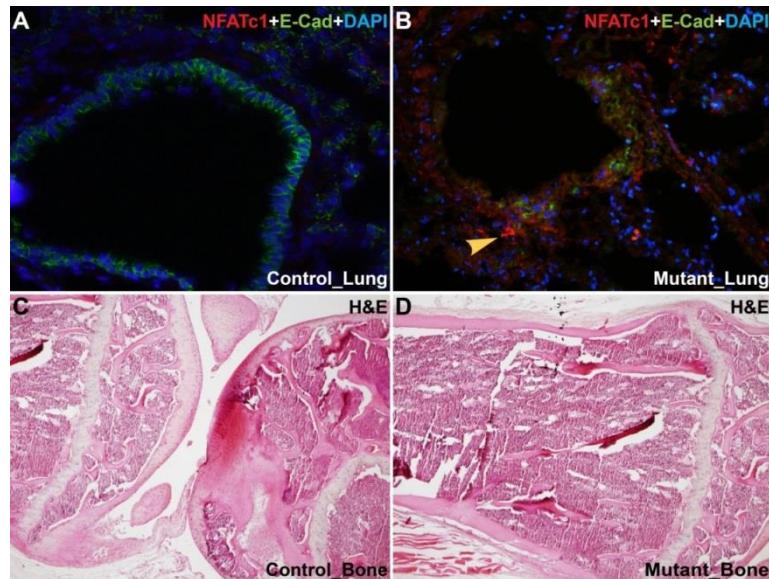


Figure 13: No evidence of metastasis to other organs has been found in mice carrying NFATc1-induced PCa with additional PTEN deficiency for a relatively short period of time. Unexplained appearance of NFATc1+ cells in lung tissue in some mutants was noted (B). But these are not considered signs of metastasis since there was not E-Cad+ NFATc1+ cells found.

Major Task 3: Study the NFATc1-induced tumorigenic microenvironment and the role of SPP1 in prostate cancer

3:1. We further analyzed the cellular & molecular components of the prostate cancer microenvironment

Molecular Components of tumorigenic microenvironment:

We hypothesize that, in addition to cell autonomous effects, NFATc1-induced transcriptional changes in the prostatic epithelium directly and indirectly lead to the production of secreted factors, including SPP1 (a reported NFAT target and a key prognostic marker for prostate cancer that is upregulated in the NFATc1-induced prostate cancer), capable of establishing a permissive/conducive microenvironment for prostate cancer development. To test this hypothesis, we have investigated changes in some key cellular and molecular components in this tumorigenic microenvironment as a result of NFATc1 activation in the prostatic epithelium. After harvesting the prostate samples from control and mutant mice (capable of NFATc1 activation in the prostatic epithelium under Dox treatment), total RNA was isolated by using the Trizol reagent (Life Technologies) and purified with an RNeasy Mini Kit (Qiagen).

cDNA was prepared from RNA using the Invitrogen ThermoScript™ RT-PCR System (Life Technologies). We have found upregulation of a range of cytokines and secreted factors implicated in cancer progression. Some of these are shown in Figure 14, including SAA1, SAA3, SPP1, CCL3, CCL4, S100A8, IL6, IL1 α , IL1 β , and others at transcriptional levels.

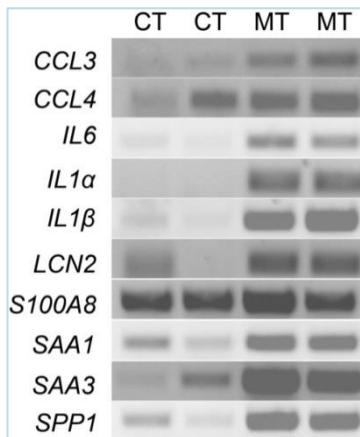


Figure 14: Molecular changes in the tumor microenvironment. A number of secretory factors known to play a role in prostate cancer progression like, SPP1, IL6, and IL1, along with several other genes were evaluated for transcriptional changes with RT-PCR using RNA from prostate of control (CT) and mutant (MT) mice treated with Dox for ~14 weeks.

Direct upregulation of cytokines and secreted factors by NFATc1 activation

The above data and some of the immunofluorescence staining results on tissue sections (Fig. 15 and data not shown) have clearly shown the increased presence of multitudes of cytokines and other secreted factors in the prostate cancer microenvironment. To clarify if the upregulation of these factors is a result of increased production of them in cells with NFATc1 activation or as secondary responses, we studied the expression changes of selected factors in isolated cancer cells, outside of the in vivo environment, to see if NFATc1 activation directly causes these cells to produce more cytokines and other secreted factors. For primary tumor cell culture, prostate from 14-week-old mutants (PCre/+;RT+/+;TetO-NFATc1^{Nuc}) were harvested and cut into small pieces of <1mm and cultured in Dulbecco's Modified Eagle's Medium-F12 (10% FBS, 5% penicillin/streptomycin and 2 μ g/ml Dox). Cells grew out of the tumor tissue chunks were fed with fresh media every 2-3 days, and sub-cultured before confluence. Cultured cells were further purified by clonal selection. Colonies exhibiting epithelial cell morphology were isolated by using clonal rings, trypsinized and sub-cultured. One of the clonal lines was further expanded and used for cell culture studies. Cultured cells were tested for nuclear NFATc1 expression in the presence of Dox.

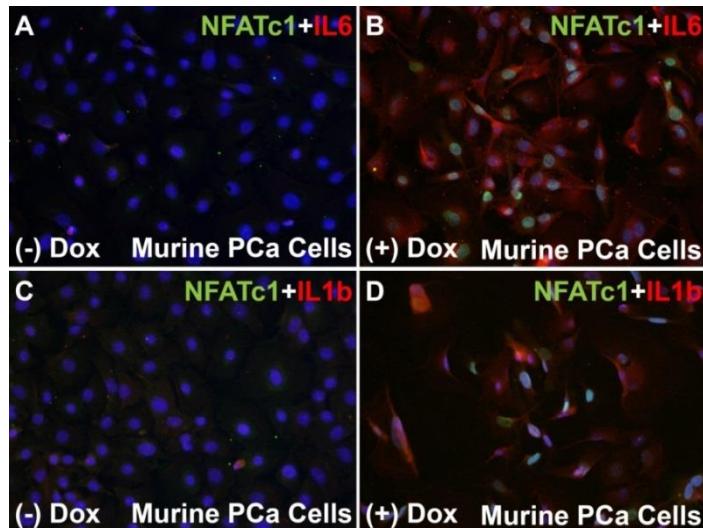


Figure 15: Increased expression of IL6 and IL1 β in murine prostate cancer cells as a direct result of NFATc1 activation.

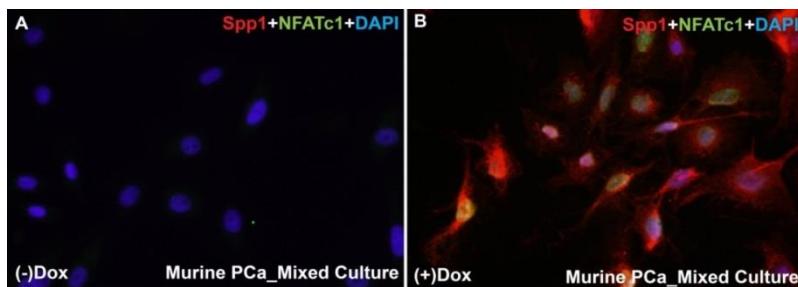


Figure 16: NFATc1 drives SPP1 expression in murine PCa cells. The murine PCa cells we generated from NFATc1-induced PCa do not express SPP1 without Dox treatment. When treated with Dox, these cells showed NFAT activation and greatly increased Spp1 expression (A-B).

Multiple recent studies have collectively indicated that SPP1 is one of the four key signatures genes correlated with prostate cancer progression and prognosis.²⁸ Thus, we have also examined if the production of SPP1 in these murine tumor cells are NFATc1 dependent. As shown in Figure 16, SPP1 upregulation is seen in Dox-treated cells, but not in the non-treated cells, demonstrating again a strong correlation between NFATc1 activation and the increased production of SPP1. This is consistent with some earlier reports that NFATc1 may regulate SPP1.²⁹

We have demonstrated in these experiments that the increased expression of multiple cytokines and other secreted factors are a direct result of NFATc1 upregulation in the prostatic epithelial cells. The upregulation of these factors are key events in the further establishment of a proinflammatory and promitogenic microenvironment that will affect cellular behavior.

Cellular components of tumorigenic microenvironment

In addition to delineating the key molecular components of this tumorigenic microenvironment, we have also revealed some the key cellular components of the inflammatory environment by studying local and infiltrating cells. We found extensive infiltration of CD3+ T cells (Figure 16A-B) in the NFATc1-induced prostate cancers. Along with T cells, we also found significant number of F4/F80⁺ macrophages (Figure 16C-F) in prostate cancer but not the control samples after 3 months of NFATc1 activation. Immune cell infiltration occurs after 2 days, since at that time, no significant increase of macrophage (Figure 16C-D) or T cells (not shown) has occurred. The exact timing or the infiltration and the potential presence of other cell types will be further studied.

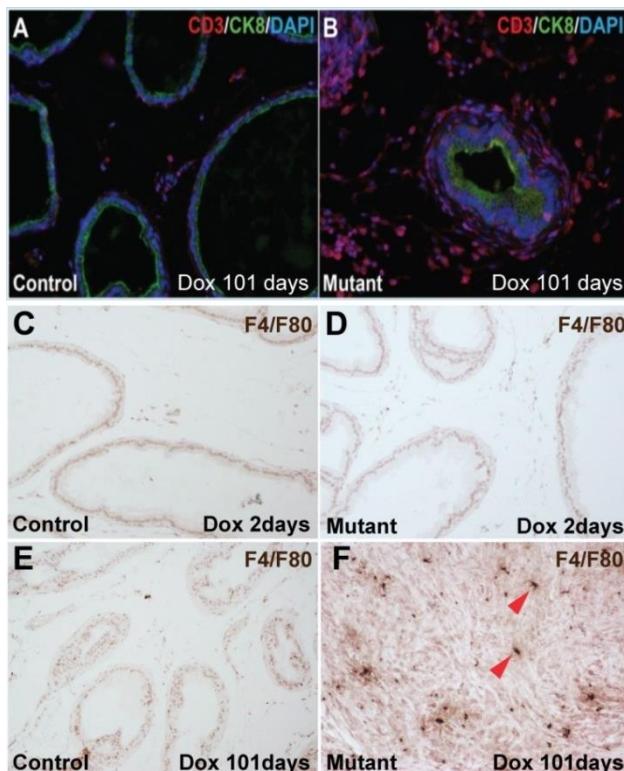


Figure 17: Infiltration of immune cells is an important process in the establishment of a pro-inflammatory and pro-mitogenic microenvironment. Extensive infiltration of CD3+ T cells in prostate cancers in the Dox-treated mutants when compared to control prostate (A-B). Significant number of F4/F80⁺ macrophages was present in prostate cancers in mutants treated with Dox for 14 weeks but not in the controls. This appears to be a late event since prostates from both controls and mutants treated with Dox for 2 days did not show any macrophage infiltration (C-F).

Wnt signaling is thought to be important in prostate cancer, in part because proteins such as β -catenin can also affect androgen receptor signaling.³⁰⁻³⁶ Besides being part of a cell adhesion complex with E-cadherin, β -catenin is also an essential component in transducing Wnt signaling on the cell membrane to the transcriptional responses in the nucleus through the canonical Wnt signaling pathway. A number

of studies have reported the altered expression and/or localization of β -catenin as a biomarker in prostate cancer. NFAT can activate COX2, c-Myc, Wnt, Frizzled, SFRP2 and others to cause increased cell migration, metastasis, and angiogenesis. Wnt activates its canonical β -catenin-TCF pathway for signal transduction and transcription in the nucleus. Non-canonical Wnt pathway can also activate NFATc1 pathway, causing expression of several Wnt responsive genes. We wanted to determine if NFATc1-induced prostate cancer has an alteration in β -catenin expression. We found upregulation of β -catenin in NFATc1-induced prostate cancers when compared to control (Fig. 18). The exact effects of β -catenin upregulation in the progression of NFATc1-induced prostate cancer are still being investigated.

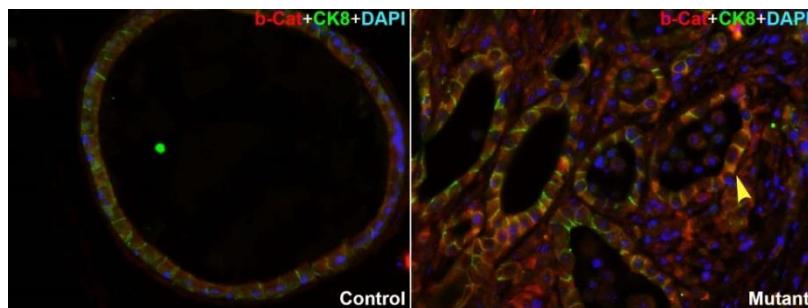


Figure 18: NFATc1 activation upregulates β -catenin, an important mediator of canonical Wnt signaling. CK8+ cells in normal murine prostates do not show any β -catenin expression whereas murine NFATc1-induced prostate cancers showed upregulated β -catenin expression.

3.2 Study the role of SPP1, an NFATc1 target, in NFATc1-induced prostate cancer.

SPP1 has been indicated as a key biomarker for prostate cancer.²⁸ Previous experiments have also shown that SPP1 can be activated by NFAT signaling and serves as a major downstream factor of the NFAT effects in smooth muscle cells.³⁷ By using the cultured cell line we generated from the NFATc1-induced murine prostate cancer, we showed that SPP1 is upregulated in the NFATc1 positive cells (Fig. 19).

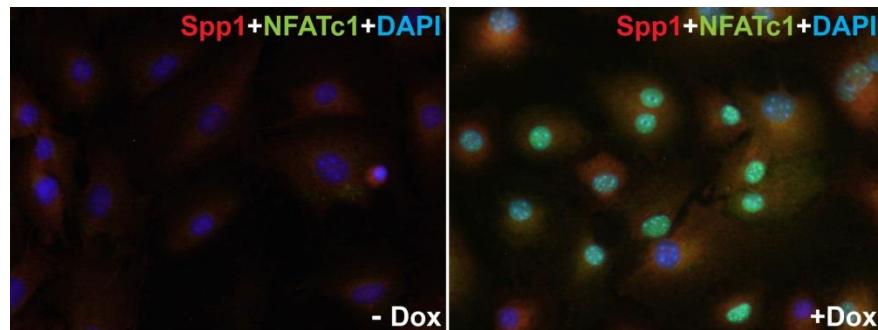


Figure 19: Removal of Dox diminishes Spp1 expression in murine prostate cancer cells.

We have generated some of the mice necessary to evaluate the importance of SPP1 in the oncogenic effects of NFATc1 in prostate cancer through studying the effects of inactivating SPP1 in mice with NFATc1 activation in prostatic epithelium and the in vivo effects of upregulation of SPP1 in the prostate for prostate cancer development, with or without NFATc1 activation in the prostatic epithelium. We have generated the tetO-SPP1 transgenic mice (Tsp^{+/}) mice. Tsp^{+/} mice are viable and fertile and live normal without any obvious defects. By combining the PCre (Probasin-Cre) transgene, the ROSA-rtTA allele, and the Tsp transgene, we have generated some mutants capable of Dox-mediated upregulating SPP1 in prostatic epithelium (PCre^{+/}; ROSA-rtTA, Tsp^{+/}) and controls that cannot upregulate SPP1 even in the presence of Dox. The expression of SPP1 in these was evaluated as revealed by immunofluorescence (Fig. 20). Dox-treated mutant mice showed elevated expression of SPP1 in and near the CK8⁺ prostatic epithelium whereas control mice did not show any significant signals.

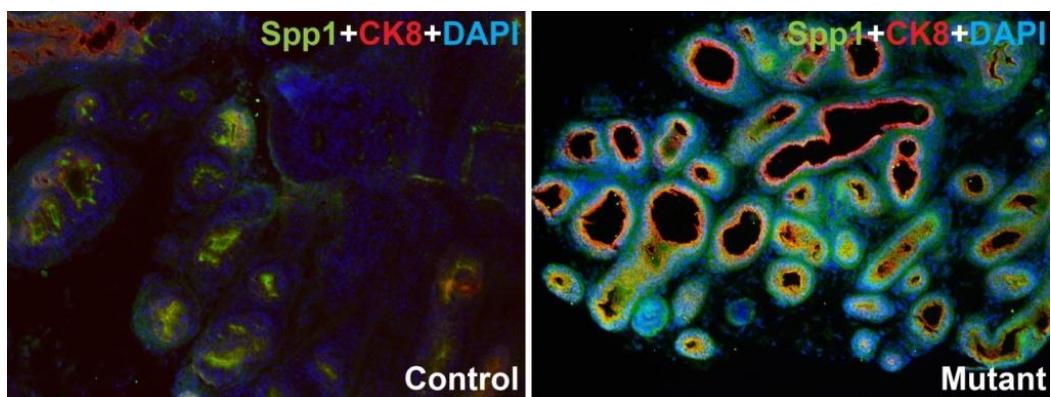


Figure 20: Upregulation of Spp1 in the murine prostate by an inducible transgene.

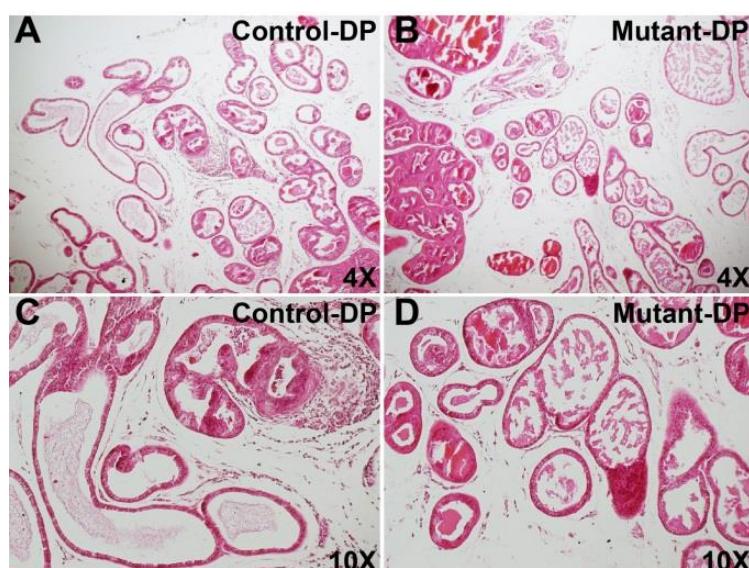


Figure 21: Transgenic upregulation of Spp1 in prostatic epithelium alone did not initiate PCa.
DP: Dorsal prostate.

The mutants and controls treated with Dox were monitored and analyzed for tumor formation for 12 weeks (Fig. 21). The prostates from either the control or the mutants did not show any evident of abnormalities. There is no sign of prostatic intraepithelial neoplasia (PIN) or cancer in prostates even after 12 weeks. This is in contrast to NFATc1 mutant mice that develop prostatic adenocarcinoma at 12 weeks of age. These results suggest that SPP1 upregulation by itself is not sufficient to initiate tumorigenesis in the prostate. It may however, play a key role in the progression of PCa as some previous reports suggested.²⁸

In addition, we have generated some mice that are PCre/+; ROSA-rtTA/+; TetO-NFATc1^{Nuc}/+; TetO-Spp1/+-. These mice will have NFATc1 activation and SPP1 upregulation when treated with Dox. While we are still in the process of generating more of the control and mutant groups to perform statistically significant analyses, we will start to treat the initial batch of these mice with Dox and monitor for tumor formation. In parallel, we are generating PCre/+; ROSA-rtTA/+; TetO-NFATc1^{Nuc}/+; Spp1^{ff} for NFATc1 activation and germ line SPP1 inactivation to determine if the NFATc1 oncogenic effects will be diminished by the inactivation of SPP1, one of the factors greatly increased in the NFAT-induced prostate cancer and one of the key markers for prostate cancer progression.²⁸

Major Task 4: Determine if there is a direct connection between NFATc1 expression and human prostate cancer pathogenesis

4.1: We performed initial study of a potential connection between NFATc1 expression and human prostate cancer progression

Our analyses using NFATc1 antibody to stain sections from human prostate cancer samples found NFATc1+ cells in the neoplastic epithelium in 18 (~30%) of the adenocarcinoma specimens (n = 57) with Gleason scores ranging from 5–9, but not in the epithelium of non-neoplastic prostates (n = 30). We collected some additional samples adenocarcinoma and found ~30% of total samples had NFATc1 expression.

We have analyzed a small number of human metastatic prostate cancer samples, mainly from lymph nodes (n=11) and bone metastases (n=12). We use immunostaining on sections to look for NFATc1 and E-Cad double positive cells (Fig. 22). Almost all of the samples had NFATc1+ cells. We have so far no convincing evidence that any of these NFATc1+ cells are metastatic cancer cells, since most are E-Cad negative. We are continuing our efforts by scanning through more areas of the existing samples

and acquiring more human specimens to increase the sample size and determine if NFATc1 activation is necessary to metastases of PCa.

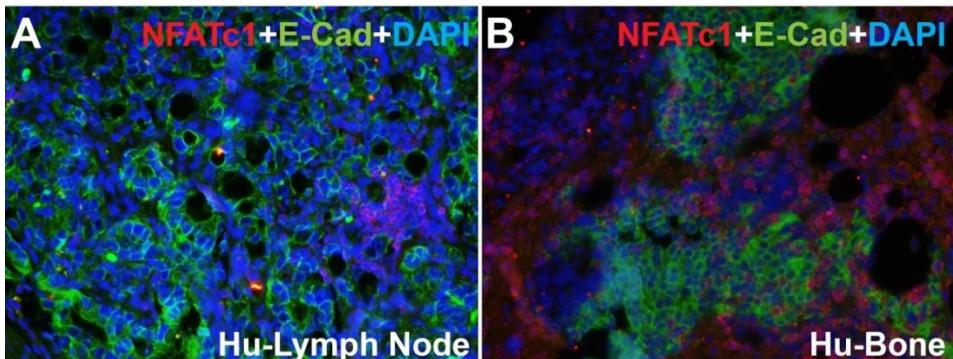


Figure 22: Absence of NFATc1 and E-Cad double positive cells in lymph nodes and bone metastasis specimens from human PCa patients.

4.2 Investigate the oncogenic effects of NFAT signaling in human prostate cancer cell lines

We have shown no NFATc1 expression in the prostatic epithelial cell line RWPE1 that is immortalized but not tumorigenic. On the other hand, the metastatic prostate cancer-derived PC3 and DU145 cells have extensive NFATc1 expression and concurrent SPP1 expression.¹¹ In the next set of experiments, we set out to determine if the modulation of NFAT activity using different inhibitors can alter cellular behavior related to tumorigenesis and cell migration, an important step in cancer metastasis.

The first in vitro assay we did was the clonogenic assay or colony formation assay. We compare the ability of different cell lines to form colonies in the presence or absence of NFAT activation or inhibition (Fig. 23). Non-transformed prostate epithelial cells RWPE-1 did not express NFATc1 and did not form colonies after 3 weeks. A large percentage of PC-3 cells and LNCap cells have NFATc1 expression. These lines can form colonies I soft agar during the same period of time. The cell line we generated from the NFATc1-induced prostate cancer forms colonies only in the presence of Dox that induces NFATc1 activation, but not in the absence of Dox. These data, especially the results from the cancer cell line we generated, suggest that NFATc1 may promote anchorage-independent growth and transformation.

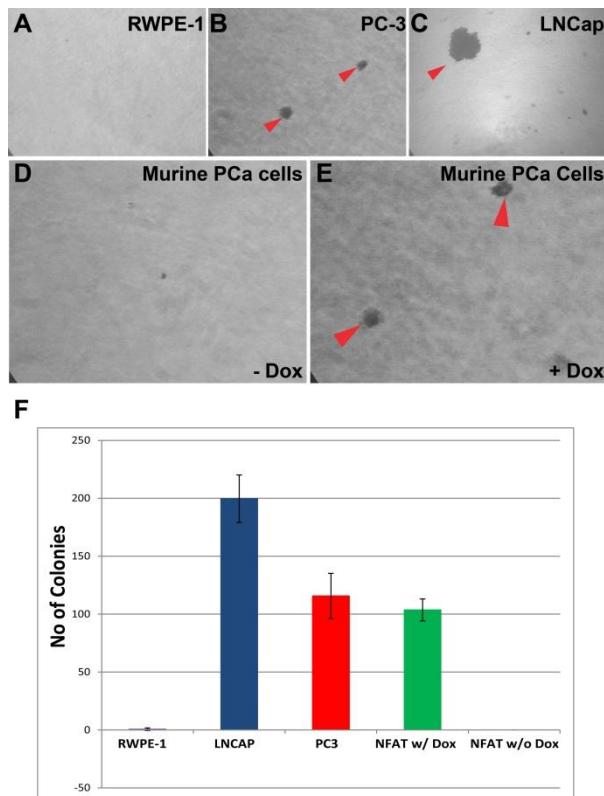


Figure 23: Colony formation correlates to NFAT activity. A, Non-transformed prostate epithelial cells RWPE-1 did not form colonies after 3 weeks. B-C, PC-3 cells and LNCap cells form colonies. D-E, the cell line we generated from the NFATc1-induced prostate cancer forms colonies only in the presence but not in the absence of Dox. E, Quantification of the results. P <0.05 when LNCaP, PC3, and “NFAT w/ Dox” were compared to the RWPE-1 data. P <0.05 when “NFAT w/ Dox” and “NFAT w/o Dox” are compared.

To examine if the NFAT activity may affect cell migration, we performed an in vitro scratch wound healing assay and a Boyden chamber transwell invasion assay using the PC3 cells in the presence or absence of NFAT inhibitors. In the wound healing assay, fully confluent PC3 cells in 6 well plates were scratched and allowed to heal in the presence or absence of NFAT inhibitors Cyclosporine A (1uM) and VIVIT (2uM) respectively (Fig. 24). While mock-treated cells migrate to close the gap significantly 48 hours after the scratches were made, very little has changed in the wells where calcineurin-NFAT inhibitors (Cyclosporine and VIVIT) were present.

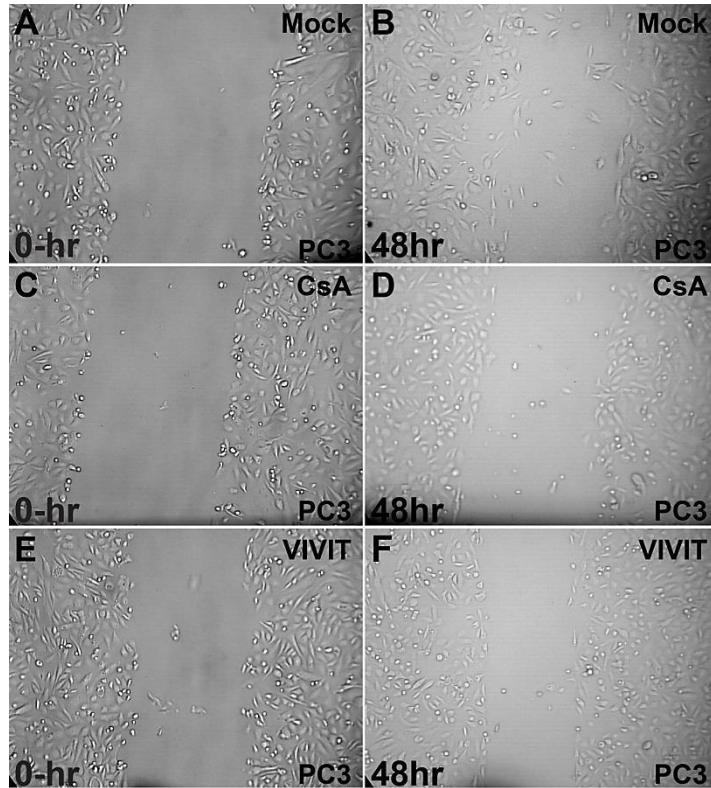


Figure 24: Inhibition of NFAT prevents/delays scratch wound healing in cultured PC3 cells that express NFATc1. While mock-treated cells showed significant wound closure after 48 hours (A-B), NFAT inhibition by cyclosporine (C-D) and VIVIT (E-F) negatively impacts the migration of PC3 cells in this wound healing assay.

Similarly, in the Boyden chamber assay, CsA and VIVIT treatment demonstrated marked decreases in the number of PC3 cells migrated across the membrane (Fig. 25A-B). On the contrary, more ionomycin-treated cells have migrated when compared to mock-treated cells (Fig. 25C-D). Average numbers of cells migrated through the membrane per well ($n=3$) were 262 ± 31.32 in mock-treated cells (Fig. 25E). This number is 347 ± 23.24 in ionomycin (a Cn-NFAT activator) -treated cells, significantly higher than that of the untreated sample. On the contrary, the numbers of the migrated cells were significantly smaller than the mock-treated control when calcineurin and NFAT inhibitors (CsA: 134 ± 5.56 cells and VIVIT: 130 ± 17.05 cells) were used. These results are consistent with the scratch wound healing results, pointing to a link between NFAT activity and cell migration in these prostate cancer cells. Taken together, results from all the in vitro assays (colony formation, wound healing, and Boyden chamber) appear to indicate that NFAT signaling promotes the transformation, anchorage-independent growth, and migration. Inhibiting this pathway may disrupt and/or reverse some of these processes associated with cancer initiation and progression.

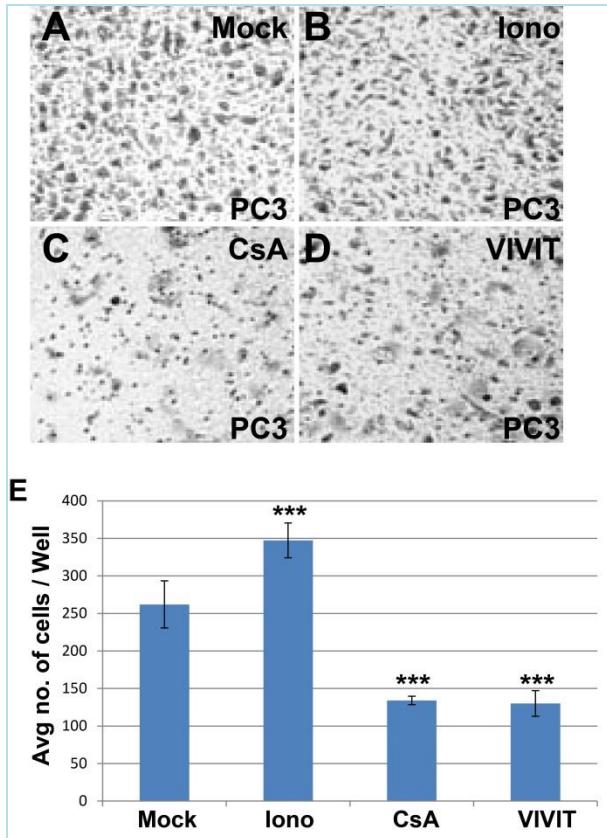


Figure 25: NFAT inhibition affects migration of tumor cells. Inhibition of NFAT in PC3 cells by cyclosporine (C) and VIVIT (D) prevents migration of cells in a Boyden chamber assay whereas ionomycin increases migration when compared mock treated cells (A-B). *** P ≤0.001 when compared to the mock-transfected cells.

We performed two sets of RNA-Seq analyses to further study the molecular mechanism of NFAT induced tumorigenesis. In the first set, we compared transcriptional profile of mouse PCa cells with or without Dox-induced NFATc1 activation. 87 genes have significant upregulation while 170 genes have significant down regulation within a day of treatment (Fig. 26) We found that, after only 24 hours, a number of genes were upregulated for over 3 folds by NFATc1 activation (Fig. 27). These may be the immediate transcriptional targets of NFATc1 in prostate epithelial cells. In a second set of experiments, we compared the expression profiles of PC3 PCa cells with or without FK506 (a Calcineurin-NFAT Inhibitor) treatment for 24 hours (Fig. 28). A number of genes were already suppressed by this calcineurin-NFAT inhibitor. These findings will help to further understand the molecular regulation by calcineurin-NFAT in prostatic epithelial cells and the effects of alteration in this pathway.

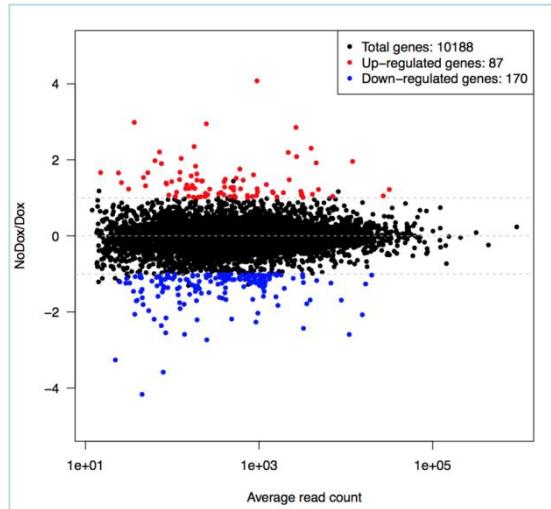


Figure 26: NFATc1 activation changed gene expression, as revealed by RNA-Seq.

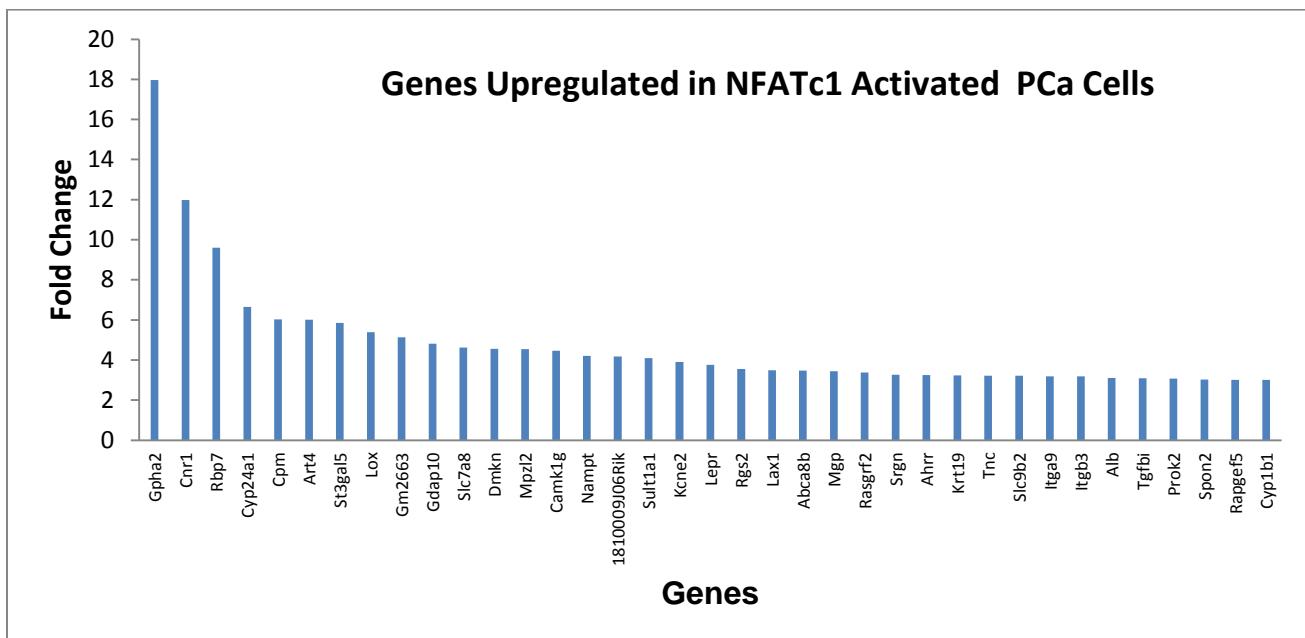


Figure 27: NFAT activation upregulates the expression of a set of genes in PCa cells.

The genes in the chart have over 3 fold upregulation within 24 hours after the activation of NFATc1 in cultured PCa cells treated with Dox to induce NFATc1.

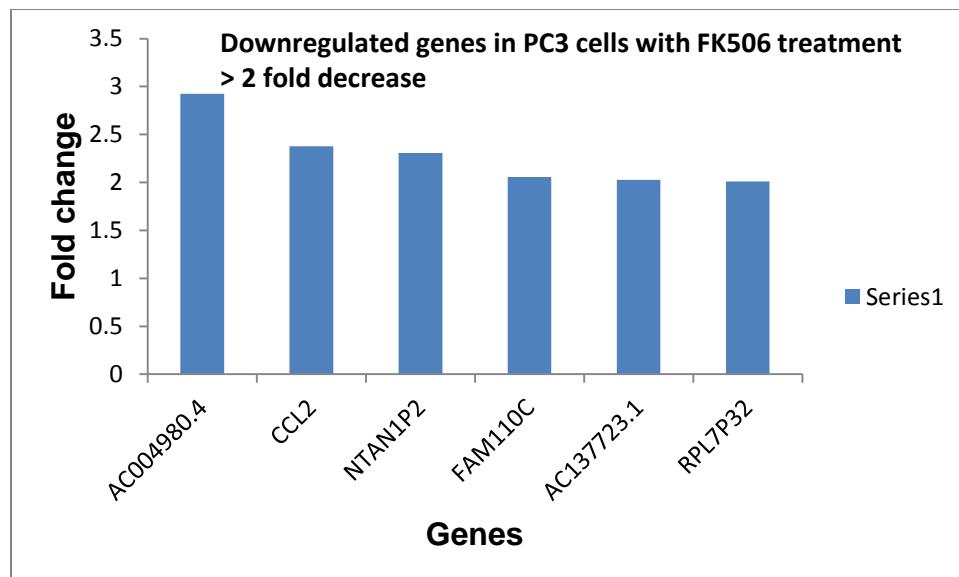


Figure 28: NFAT inhibition down-regulates the expression of a set of genes in PCa cells.

Inhibition of Cn-NFAT in PC3 cells by FK506 leads to the downregulation of specific genes in 24 hours.

Key citation: Manda KR, Tripathi P, Hsi AC, Ning J, Ruzinova MB, Liapis H, Bailey M, Zhang H, Maher CA, Humphrey PA, Andriole GL, Ding L, You Z, Chen F: NFATc1 promotes prostate tumorigenesis and overcomes PTEN loss-induced senescence, Oncogene 2016, 35:3282-3292

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4) Other achievements.

During the grant period, besides the above work that is directly related to the funded project, we have performed other studies. Because efforts of the PI (Feng Chen) and the collaborator (Zongbing You) were partially funded by this award, we acknowledged this award in our following publications. In some earlier papers, Dr. You put the award number in the acknowledgement of his papers without mentioning Dr. Chen as the PI, partly because Dr. You is the PI of a subaward based on this award. However, after the first funding period and

recommendations we received from reviewers and science officers, Dr. You has been putting “(PI: Feng Chen; Co-I: Zongbing You)” in his publications to avoid any misunderstanding.

(1) Beifang Niu*, Adam D. Scott*, Sohini Sengupta (* Co-first authors), Matthew H. Bailey, Prag Batra, Jie Ning, Matthew A. Wyczalkowski, Wen-Wei Liang, Qunyuan Zhang, Michael D. McLellan, Sam Q. Sun, Piyush Tripathi, Carolyn Lou, Kai Ye, Robert J. Mashl, John Wallis, Michael C. Wendt, **Feng Chen**#, and Li Ding# (# Co-Corresponding Authors)
Protein structure guided discovery of functional mutations across 19 cancer types

Nature Genetics 2016 Aug; 48(8):827-37. PMID: [27294619](#)

Status of publication: published; acknowledgement of federal support: Yes.

(2) Song Cao, Michael C. Wendt, Matthew A. Wyczalkowski, Kristine Wylie, Kai Ye, Reyka Jayasinghe, Mingchao Xie, Song Wu, Beifang Niu, Robert Grubb III, Kimberly J. Johnson, Hiram Gay, Ken Chen, Janet S. Rader, John F. Dipersio, **Feng Chen**, and Li Ding (2016)
Divergent viral presentation among human tumors and adjacent normal tissues

Science Reports 6, 28294.

Status of publication: published; acknowledgement of federal support: Yes.

(3) Kai Ye, Jiayin Wang, Reyka Jayasinghe, Eric-Wubbo Lameijer, Joshua F. McMichael, Jie Ning, Michael D. McLellan, Mingchao Xie, Song Cao, Venkata Yellapantula, Kuan-lin Huang, Adam Scott, Steven Foltz, Beifang Niu, Kimberly J. Johnson, Matthijs Moed, P. Eline Slagboom, **Feng Chen**, Michael C. Wendt, Li Ding (2016)

Systematic Discovery of Complex Indels in Human Cancers

Nature Medicine 22(1):97-104. PMID: 26657142

Status of publication: published; acknowledgement of federal support: Yes.

(4) S. Liu, Q. Zhang, C. Chen, D. Ge, Y. Qu, R. Chen, Y. M. Fan, N. Li, W. W. Tang, W. Zhang, K. Zhang, A. R. Wang, B. G. Rowan, S. M. Hill, O. Sartor, A. B. Abdel-Mageed, L. Myers, Q. Lin and **Z. You**

Hyperinsulinemia enhances interleukin-17-induced inflammation to promote prostate cancer development in obese mice through inhibiting glycogen synthase kinase 3-mediated phosphorylation and degradation of interleukin-17 receptor. *Oncotarget*, Volume: 7, Issue: 12, Pages: 13651-66, Epub Date: 2016/02/13, PMID: 4924668

Status of publication: published; acknowledgement of federal support: Yes.

(5) Zhang Q, Liu S, Parajuli KR, Zhang W, Zhang K, Mo Z, Liu J, Chen Z, Yang S, Wang AR, Myers L, **You Z**. Interleukin-17 promotes prostate cancer via MMP7-induced epithelial-to-mesenchymal transition. *Oncogene*. 2016 Jul 4. doi: 10.1038/onc.2016.240.

PMID: 27375020.

Status of publication: published; acknowledgement of federal support: Yes.

(6) Qu Y, Zhang Q, Ma S, Liu S, Chen Z, Mo Z, **You Z**. Interleukin-17A Differentially Induces Inflammatory and Metabolic Gene Expression in the Adipose Tissues of Lean and Obese Mice. *Int J Mol Sci*. 2016 Apr 7;17(4):522. doi: 10.3390/ijms17040522.

PMID: 27070576

Status of publication: published; acknowledgement of federal support: Yes.

(7) Pre-cancerous clonal expansion, a new therapeutic target? (2017)

Li Ding and **Feng Chen**

Journal of Clinical Oncology 35, no. 14 1503-1505. PMID: [28291387](#)

Status of publication: published; acknowledgement of federal support: Yes.

What opportunities for training and professional development has the project provided?

Postdoctoral and other researchers involved in this project were partially supported by this funding. These researchers have gained substantial training in animal disease models and cancer biology as a result of their participation in this research.

How were the results disseminated to communities of interest?

Results have been mainly communicated through scientific publications and meetings at this time.

What do you plan to do during the next reporting period to accomplish the goals?

The major goals of this study have been accomplished and the funding period ended on 9/29/2017. While we will continue to study the roles of calcineurin-NFAT in tumorigenesis, these ongoing and future studies will not be covered by this DoD grant.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

A major challenge for prostate cancer diagnosis/prognosis and treatment is the lack of reliable biomarkers and effective therapeutic targets. In recent years, we have witnessed vigorous debates about the effectiveness and side effects of various prostate cancer screening methods, including the measurement of prostate specific antigen (PSA). It is clear that no single existing marker by itself is sufficient to provide reliable diagnostic/prognostic values and more biomarkers need to be studied to establish an informative matrix to evaluate patients risk and to distinguish aggressive from indolent diseases in prostate cancer. We have shown upregulation of NFATc1 in human prostate cancer specimens and cells. We have also provided the first direct *in situ* evidence in mice that NFATc1 activation induces prostate cancer resembling human prostate cancer. The proposed study is built on these findings and the versatile disease models we generated to further investigate prostate cancer pathogenesis, aiming at revealing the molecular network regulated by NFAT in prostate cancer and the

complex interplay between cancer cells and their microenvironment. Successful completion of this study will present NFATc1 and related molecules as potential diagnostic/prognostic markers and novel therapeutic targets in prostate cancer. These results will also enhance our understanding of the regulation of PTEN & SPP1, two well-established important factors in human prostate cancer.

What was the impact on other disciplines?

There are more and more evidence that NFATc1 is an oncogene. Studies from pancreatic cancers and many other types of cancers have shown the effects of NFATc1 activation in cancer progression. Our analyses of the cell autonomous and non-cell autonomous functions of NFATc1 in prostate cancer initiation and progression provide a detailed mechanistic explanation of the effects of NFATc1 activation on downstream targets important for cancer development.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

The current funded project is basic research in nature. The primary goal is to better understand the tumorigenic mechanism in prostate cancer with a specific emphasis on NFAT pathway and microenvironment. Future studies will aim at the translational aspects of the findings to benefit the society directly.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications.

The following manuscript/paper was the most relevant.

Manda KR, Tripathi P, Hsi AC, Ning J, Ruzinova MB, Liapis H, Bailey M, Zhang H, Maher CA, Humphrey PA, Andriole GL, Ding L, You Z, Chen F: NFATc1 promotes prostate tumorigenesis and overcomes PTEN loss-induced senescence, Oncogene 2016, 35:3282-3292

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers, and presentations.

During the grant period, besides the above work that is directly related to the funded project, we have performed other studies. Because efforts of the PI (Feng Chen) and the collaborator (Zongbing You) were partially funded by this award, we acknowledged this award in our following publications. In some earlier papers, Dr. You put the award number in the acknowledgement of his papers without mentioning Dr. Chen as the PI, partly because Dr. You is the PI of a subaward based on this award. However, after the first funding period and recommendations we received from reviewers and science officers, Dr. You has been putting "(PI: Feng Chen; Co-I: Zongbing You)" in his publications to avoid any misunderstanding.

(1) Beifang Niu*, Adam D. Scott*, Sohini Sengupta (* Co-first authors), Matthew H. Bailey, Prag Batra, Jie Ning, Matthew A. Wyczalkowski, Wen-Wei Liang, Qunyuan Zhang, Michael D. McLellan, Sam Q. Sun, Piyush Tripathi, Carolyn Lou, Kai Ye, Robert J. Mashl, John Wallis, Michael C. Wendl, **Feng Chen[#]**, and Li Ding[#] (# Co-Corresponding Authors)
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(7) Pre-cancerous clonal expansion, a new therapeutic target? (2017)

Li Ding and **Feng Chen**

Journal of Clinical Oncology 35, no. 14 1503-1505. PMID: [28291387](#)

Invited Lecture:

Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Cell lines: Murine prostate cancer cells with inducible NFATc1 expression.

Mice: Tbx18-Cre transgenic line, tetO-Spp1 transgenic line

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name:	Feng Chen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2307-7954
Nearest person month worked:	2
Contribution to Project:	Dr. Chen was responsible for setting project directions, for administration, supervision of laboratory staff, providing technical help to researchers, organizing analyses, and preparing reports and manuscripts.
Funding Support:	Not Applicable
Name:	Kalyan Manda
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0001-9666-1759
Nearest person month worked:	8
Contribution to Project:	Perform experiments, analyze data, prepare manuscript.
Funding Support:	Not Applicable
Name:	Piyush Tripathi
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0001-8337-9316
Nearest person month worked:	5
Contribution to Project:	Perform experiments, analyze data, prepare manuscript.
Funding Support:	Not Applicable

Name:	Rajees Varghese
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	0000-0001-8305-4490
Nearest person month worked:	1
Contribution to Project:	Perform experiments, analyze data.
Funding Support:	Not Applicable
Name:	Zongbing You
Project Role:	Collaborator/Consultant
Researcher Identifier (e.g. ORCID ID):	0000-0001-5048-2229
Nearest person month worked:	0.5
Contribution to Project:	Provide expert advice on the design and execution of the experiments. Help with data interpretation and manuscript preparation.
Funding Support:	Not Applicable

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report (not applicable).

9. APPENDICES:

None

ORIGINAL ARTICLE

NFATc1 promotes prostate tumorigenesis and overcomes PTEN loss-induced senescence

KR Manda¹, P Tripathi², AC Hsi³, J Ning^{1,3}, MB Ruzinova², H Liapis², M Bailey³, H Zhang⁴, CA Maher^{1,3,5}, PA Humphrey⁶, GL Andriole^{5,7}, L Ding^{1,3,5}, Z You⁸ and F Chen^{1,5,9}

Despite recent insights into prostate cancer (PCa)-associated genetic changes, full understanding of prostate tumorigenesis remains elusive owing to complexity of interactions among various cell types and soluble factors present in prostate tissue. We found the upregulation of nuclear factor of activated T cells c1 (NFATc1) in human PCa and cultured PCa cells, but not in normal prostates and non-tumorigenic prostate cells. To understand the role of NFATc1 in prostate tumorigenesis *in situ*, we temporally and spatially controlled the activation of NFATc1 in mouse prostate and showed that such activation resulted in prostatic adenocarcinoma with features similar to those seen in human PCa. Our results indicate that the activation of a single transcription factor, NFATc1 in prostatic luminal epithelium to PCa can affect expression of diverse factors in both cells harboring the genetic changes and in neighboring cells through microenvironmental alterations. In addition to the activation of oncogenes c-MYC and STAT3 in tumor cells, a number of cytokines and growth factors, such as IL1β, IL6 and SPP1 (osteopontin, a key biomarker for PCa), were upregulated in NFATc1-induced PCa, establishing a tumorigenic microenvironment involving both NFATc1 positive and negative cells for prostate tumorigenesis. To further characterize interactions between genes involved in prostate tumorigenesis, we generated mice with both NFATc1 activation and Pten inactivation in prostate. We showed that NFATc1 activation led to acceleration of Pten null-driven prostate tumorigenesis by overcoming the PTEN loss-induced cellular senescence through inhibition of p21 activation. This study provides direct *in vivo* evidence of an oncogenic role of NFATc1 in prostate tumorigenesis and reveals multiple functions of NFATc1 in activating oncogenes, in inducing proinflammatory cytokines, in oncogene addiction, and in overcoming cellular senescence, which suggests calcineurin-NFAT signaling as a potential target in preventing PCa.

Oncogene (2016) **35**, 3282–3292; doi:10.1038/onc.2015.389; published online 19 October 2015

INTRODUCTION

Recent discoveries in prostate cancer (PCa) research have highlighted a number of key genetic alterations in driving prostate tumorigenesis. Despite these advances, the progression from the initial genetic changes to clinically significant PCa is still not fully understood.¹ Furthermore, the interactions of various genes involved in pathogenesis of PCa needs to be further investigated.

The NFAT (nuclear factor of activated T cells) family of transcription factors are important for many diverse cellular processes including T-cell activation, cardiac valve development and osmotic stress response.^{2,3} The NFAT family includes four NFATc proteins (c1–c4) and NFAT5. The NFATc proteins reside in the cytoplasm in quiescent cells. The Ca²⁺-dependent serine/threonine phosphatase calcineurin, when activated by an increase in intracellular Ca²⁺, dephosphorylates the NFATc proteins and exposes their concealed nuclear localization signals, causing them to translocate from cytoplasm to the nucleus.^{2,3} Once in the nucleus, NFATc proteins complex with cell-type specific cofactors to control the transcription of target genes. Phosphorylation of NFATc by GSK3β and other kinases promotes nuclear export of these proteins.

NFATc1 activation was shown to induce transformation and colony formation in 3T3-L1 preadipocytes.⁴ Elevated or ectopic NFATc1 activation has also been observed in multiple types of human tumors.^{3,5} NFAT proteins have also been found to regulate proliferation and growth of multiple human tumor cells, including PCa cells.^{3,6,7} NFATc1 has been shown to regulate prostate-specific membrane antigen and genes involved in osteoclastogenesis induced by PCa cells.^{8–10} Besides growth and proliferation, components of the calcineurin-NFAT signaling pathway, including NFATc1, have also been linked to cell migration, tumor invasion and metastasis in human cancers.^{3,11–15} NFATc1 effects are not always pro-growth and some NFATc genes may act as tumor suppressors.^{3,16} NFATc1 activation can lead to cell loss and fibrosis in some contexts.^{3,17} Thus, biological consequences of NFAT activation in different tissues may be very different and the molecular mechanism by which calcineurin-NFAT affects PCa *in situ* is hard to predict and needs to be directly studied.

In this study, we generated a murine model where NFATc1 activation can be induced in prostate epithelium. The activation of NFATc1 results in prostatic intraepithelial neoplasia (PIN), which progresses to prostate adenocarcinoma. We further demonstrated that NFATc1 activation establishes a promitogenic

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microenvironment with upregulation of proinflammatory cytokines and growth factors. We have also shown that NFATc1 and the PTEN-AKT pathway act synergistically in promoting PCa, as NFATc1 activation overcomes the PTEN loss-induced cellular senescence. This study provides direct *in vivo* evidence of an oncogenic role of NFAT in PCa and offers insights into multifaceted progression from a defined transcriptional change in prostatic epithelia to prostate tumorigenesis involving both cell autonomous changes in oncogenic protein expression and the effects of secreted factors in the microenvironment.

RESULTS

NFATc1 expression is detected in human PCa specimens and PCa cells but is absent in non-neoplastic human prostates and non-tumorigenic prostatic cells.

NFATc1 expression has been previously reported in human PCa specimens.^{18–20} Using human normal prostate and PCa specimens from Biomax (Rockville, MD, USA) and from archived patient specimens, we found NFATc1⁺ cells in the neoplastic epithelium in 18 (~30%) of the adenocarcinoma specimens ($n=57$) with Gleason scores ranging from 5–9, but not in the epithelium of non-neoplastic prostates ($n=30$) (Figures 1a–c). NFATc1⁺ cells were also present in the tumor stroma. In addition, we have found NFATc1 expression in the human malignant PC3, LNCaP and DU145 cells, but not in the non-tumorigenic RWPE-1 cells (Figures 1d–g). These results are consistent with previous findings that NFATc1 expression is associated with the initiation, progression, and probably even the metastasis of the various cancers,³ including PCa.^{7,20}

Inducible NFATc1 activation in prostatic epithelium causes PIN and prostatic adenocarcinoma

To investigate the potential role of NFAT signaling in PCa, we created a mouse model for inducible NFATc1 activation in cells targeted by the *PBCre4* (*PCre*) transgene²¹ with known expression in the prostatic epithelium. In this system, Cre expression induces the removal of the transcriptional stop cassette in a *ROSA*^{rTA} (*RT*) allele²² and the production of rtTA (reverse tetracycline-controlled transactivator). In the presence of doxycycline (Dox), the Dox-rtTA complex binds to the *TetO* sequence of the *TetO-NFATc1*^{Nuc} (*TN*) transgene²³ to induce the transcription of *NFATc1*^{Nuc} (an activated form of NFATc1) (Figure 2a). We refer to mice carrying all three alleles (*PCre*, *RT*, *TN*) as mutants. Their littermates missing any of these alleles cannot have NFATc1 activation, even in the presence of Dox, and thus are regarded as controls. *NFATc1*^{Nuc} transcripts

were detected in Dox-treated mutants, but not in similarly treated controls (Figure 2b).

We treated control-mutant pairs ($n=23$) with Dox from weaning (P21-postnatal day 21) for variable lengths of time. We found a marked expansion of the prostate lobes in mutants treated for 14 weeks (Figures 2c and d). Although mutants treated for 6 weeks ($n=6$) did not show marked outward changes, histological analyses showed that they already had PIN recognized by proliferation and stratification of epithelial cells (Figures 2e and f). In total, 96% (22/23) of the mutants with NFATc1 activation for 14 weeks had PCa in the dorsolateral and ventral prostate lobes (Figures 2g and h), whereas all controls had normal epithelium. The majority of human PCa is found in the peripheral zone that is most similar to the mouse dorsolateral lobes. About half of the mutants also developed PCa in the anterior prostate. The remaining half either had PIN or failed to develop any significant neoplastic changes in the anterior prostate. The murine PCa showed an acinar growth pattern, similar to those seen in human prostatic acinar adenocarcinoma.

NFATc1-induced PCa has cellular composition resembling human prostatic adenocarcinoma

Normal prostate gland has a CK5⁺ (cytokeratin 5) and p63⁺ basal epithelial cell layer, a CK8⁺ (cytokeratin 8) luminal epithelial cell layer with AR (androgen receptor) expression, a small number of SYNAP⁺ (synaptophysin) neuroendocrine cells, and the surrounding myofibroblast cells that are α -smooth muscle actin (SMA)⁺ (Figures 3a, c, e, g and i). In mutants with NFATc1 activation for 14 weeks starting from P21, tumor expansion primarily came from CK8⁺/AR⁺ luminal epithelial cells (Figures 3b, d, f, h and j), a situation similar to the most common type of human PCa. Cytokeratin 5⁺ basal cells and SYNAP⁺ neuroendocrine cells were mostly absent from tumor proper and were found in adjacent glands that are either benign or apparently in transition to neoplasia. SMA⁺ fibromuscular layers surrounding the prostatic glands lost continuity or were absent in many areas in these mutants, likely as the result of invasion of CK8⁺ cells into the stroma (Figure 3j).

Active proliferation of NFATc1⁺ cells appears to result from a promitogenic microenvironment

In addition to a high percentage of NFATc1⁺ cells ($49.5 \pm 4.40\%$) in the PCa being PCNA⁺ (proliferating), many neighboring NFATc1[−] cells ($15.8 \pm 2.57\%$) were also PCNA⁺ (Figures 4a–c). Interestingly, although $56.6 \pm 7.45\%$ NFATc1⁺ cells were expressing c-MYC, $32.4 \pm 4.0\%$ NFATc1[−] cells also had c-MYC expression

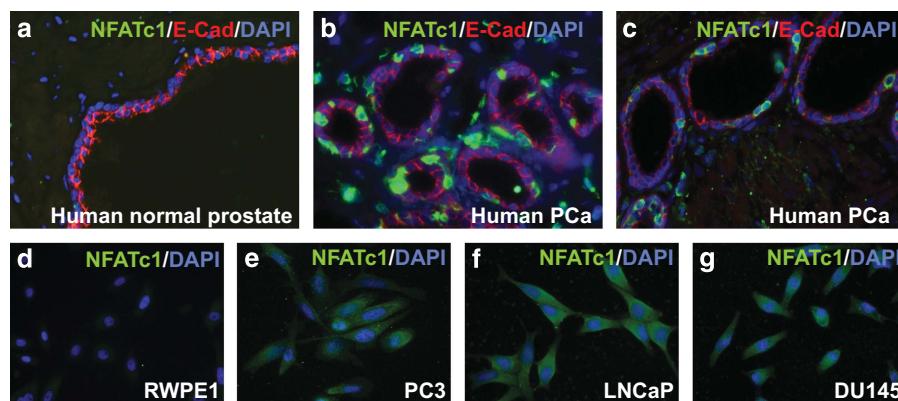


Figure 1. NFATc1 in human PCa and human PCa cell lines. NFATc1⁺ cells are absent in non-neoplastic human prostate. NFATc1⁺ cells are absent in non-neoplastic human prostate (a), but detected in human PCa (b, c). NFATc1 is expressed in the PCa cell lines but not in the non-tumorigenic RWPE-1 cells (d–g).

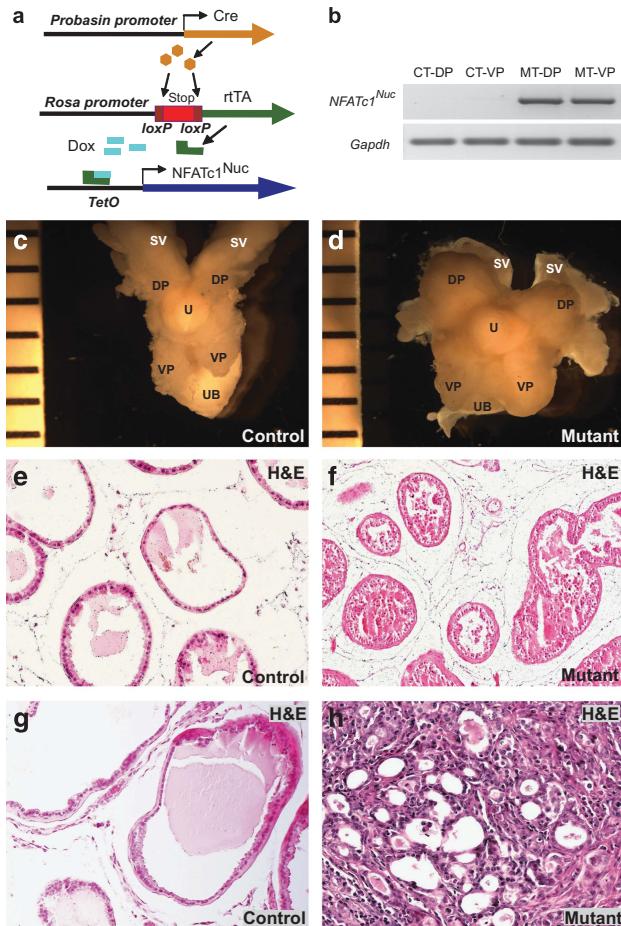


Figure 2. Inducible NFATc1 activation in prostatic epithelium causes PIN and prostatic adenocarcinoma. **(a)** Cre induces the production of rTA in prostatic epithelium. Binding of the Dox-rTA complex to TetO leads to the production of NFATc1^{Nuc}. **(b)** RT-PCR using RNA from prostates of control (CT) and mutant (MT) mice treated with Dox showed expression of NFATc1^{Nuc} only in the mutant prostates. DP: dorsal prostate. VP: ventral prostate. TetO: tetracycline-responsive operator. rTA: reverse tetracycline-controlled transactivator. **(c and d)** Prostates from control and mutant mice treated with Dox for 14 weeks starting from P21. U: Urethra; DP: Dorsal prostate; VP: Ventral prostate; UB: Urinary bladder. SV: Seminal vesicle. **(e and f)** H&E sections of the prostates from control and mutant mice treated with Dox for 6 weeks starting from P21. PINs are obvious in the mutants. **(g and h)** Prostates from control and mutant treated with Dox for 14 weeks starting from P21.

in the PCa (Figures 4d–f). Furthermore, $59.1 \pm 8.31\%$ NFATc1⁺ and $16.63 \pm 2.11\%$ NFATc1⁻ cells had nuclear phospho-STAT3 (pSTAT3) (Figures 4g–i). STAT3 activation in NFATc1⁻ cells cannot directly result from NFAT transcriptional regulation. Instead, such changes may be due to microenvironmental alterations. These observations are consistent with previous findings that cultured cells expressing an active NFATc1 secrete unidentified and heat labile factors to promote the proliferation of other cells that are NFATc1⁻.²⁴ Immunoblotting of lysates from normal prostates and NFATc1-induced PCa showed that the level of pSTAT3 was greatly increased in NFATc1-induced PCa, though total STAT3 level was unchanged (Figure 4j).

Increased expression of proinflammatory cytokines and other factors in NFATc1-induced PCa

We selected a number of cytokines and secreted factors for further analyses in the PCa based on our previous findings²⁵ and other

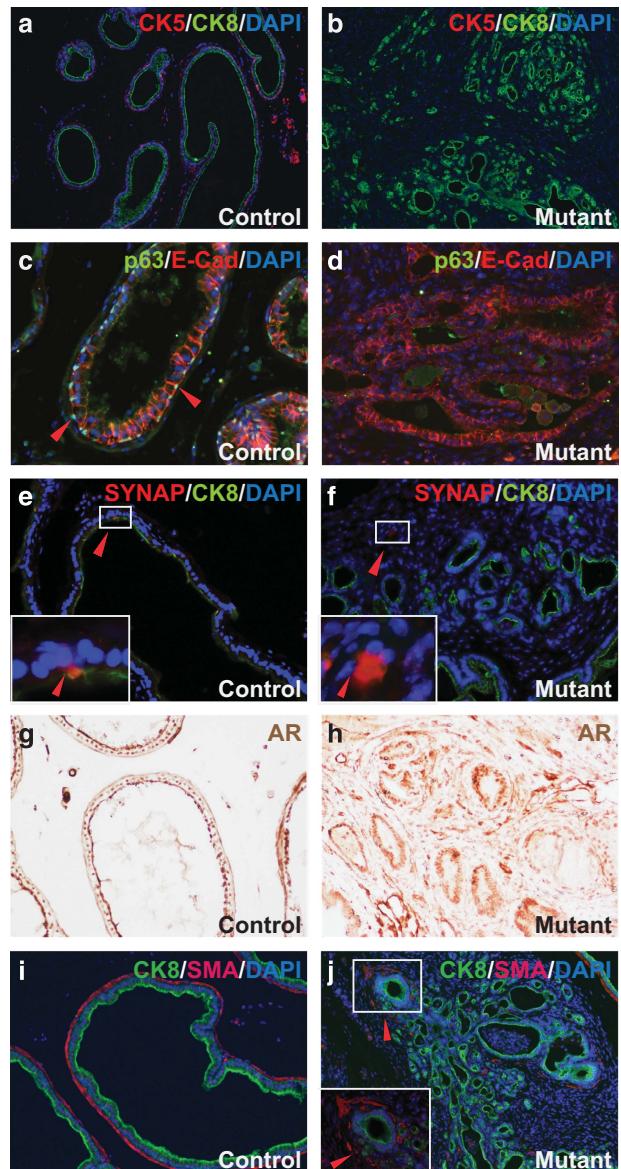


Figure 3. NFATc1-induced PCa has pathological changes resembling human PCa. Samples were from littermates treated with Dox from P21 for 14 weeks. **(a)** The control prostatic gland has CK5⁺ basal cells and CK8⁺ luminal epithelium. **(b)** The adenocarcinoma has predominantly CK8⁺ luminal epithelial cells and few CK5⁺ basal cells. **(c-d)** p63⁺ basal cells were present in the control prostate (arrowheads) but absent from NFATc1-induced PCa. **(e-f)** Very few SYNAP⁺ neuroendocrine cells are present in the periphery of the adenocarcinoma. **(g and h)** mutant luminal epithelial cells retain nuclear AR expression. Unlike the control, **(i)** discontinuation of the SMA⁺ fibromuscular layer and invasion of the CK8⁺ cells into the stroma (arrowheads in **j**) can be seen in the mutants.

studies linking them to NFAT³ and/or PCa.²⁶ By RT-PCR, we showed that NFATc1 activation was accompanied by increased levels of transcripts from Spp1, Saa3, IL6, IL1 β , IL1 α , Ccl3, Lcn2 and others (Figure 5a), some of which were implicated in promoting PCa progression.²⁷ By immunofluorescent staining, we showed significantly elevated levels of secreted cytokines, such as Spp1 and its receptor CD44, IL6 and IL1 β , in the PCa (Figures 5b–i). Spp1 has been reported as an important diagnostic and prognostic biomarker for PCa and some of the NFATc1 oncogenic effects may be channeled through the upregulation of Spp1.^{27,28}

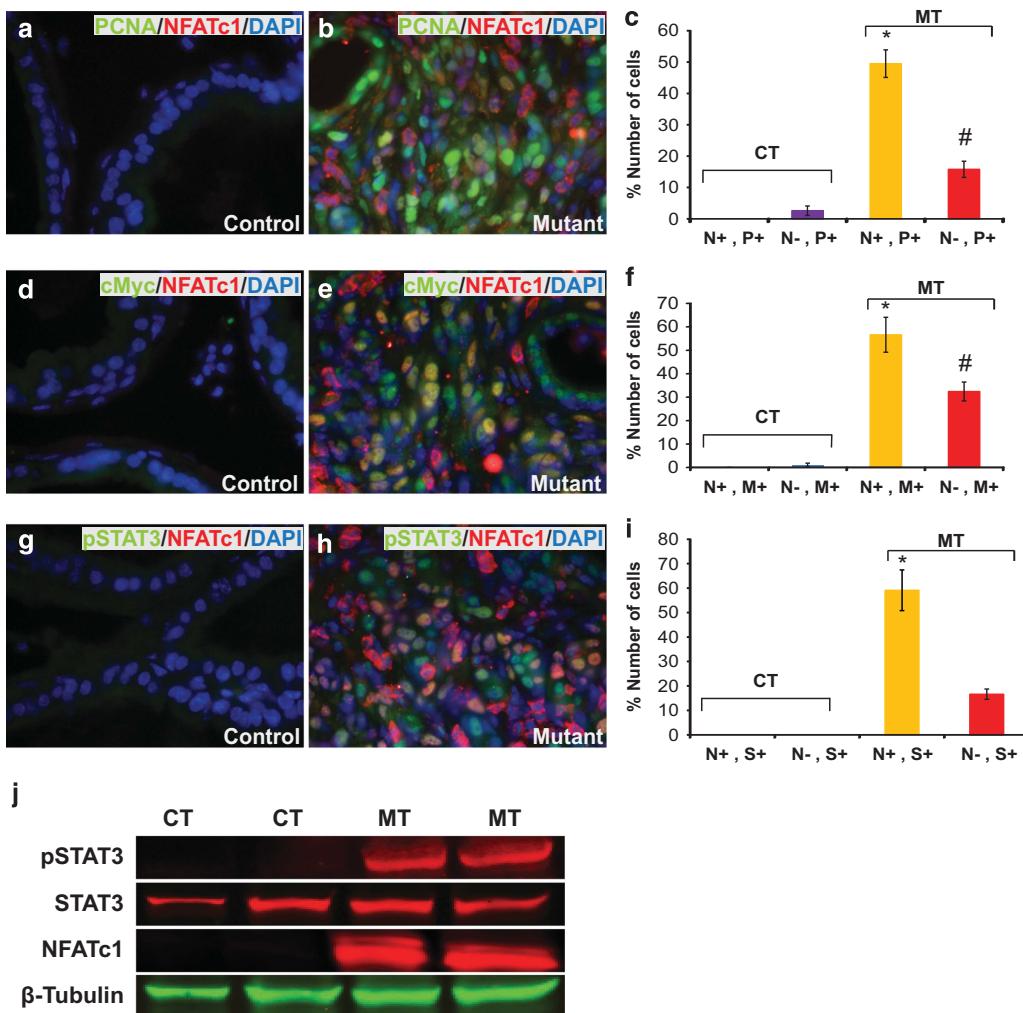


Figure 4. Active proliferation of NFATc1⁻ cells appears to result from a non-cell autonomous effects of NFATc1 activation in a promitogenic microenvironment. The prostate samples were from mice treated for 14 weeks from P21. N: NFATc1, P: PCNA, M: c-MYC, S: pSTAT3, CT: controls, MT: mutants. Immunostaining using NFATc1 and PCNA showed that a high percentage of NFATc1⁺ cells were actively proliferating ($49.5 \pm 4.40\%$). The percentage of proliferating NFATc1⁻ cells in the mutants ($15.8 \pm 2.57\%$), albeit smaller than that of the NFATc1⁺ cells in the mutants ($*P < 0.01, n=9$), was still substantial and significantly more than the NFATc1⁻ cells in the controls ($3.33 \pm 1.33\% *P < 0.01, n=9$). **(a-c)** NFATc1 activation led to significant upregulation of c-MYC in the tumor tissue. **(d-e)** $56.6 \pm 7.45\%$ of NFATc1⁺ cells expressed c-MYC and a smaller percentage ($32.4 \pm 4.0\%$) of NFATc1⁻ cells were c-MYC⁺ ($*P < 0.01, n=9$). The NFATc1⁻, c-MYC⁺ cells are drastically more numerous in the mutants than in the controls ($1.33 \pm 0.57\%, *P < 0.01, n=9$). **(f)** Double immunostaining with antibodies against NFATc1 and phospho-STAT3 (pSTAT3-Tyr705) showed higher percentage of NFATc1⁺ cells with nuclear STAT3 (%) than NFATc1⁻ cells with nuclear STAT3 ($17.67 \pm 1.45\%, *P < 0.01, n=9$). **(g-i)** However, the percentage of NFATc1⁻, pSTAT3⁺ cells is much higher in the mutants than in the control prostates ($0\%, n=9$). Western blot analysis of prostate lysates from controls and PCa from mutants was probed by antibodies against STAT3 (revealing total STAT3 levels) and pSTAT3 (revealing the levels of activated STAT3). The results showed activation of STAT3 specifically in NFATc1-induced PCa, whereas the levels of total STAT3 were unchanged. **(j)** All data are presented as mean \pm s.d. and two-tailed t-tests were performed between groups.

Tumor progression and survival depend on activation of NFATc1 but not on T-cell functions

To directly test the essential role of NFATc1 activation in tumorigenesis and to examine the involvement of lymphocytes in establishing the tumorigenic microenvironment, we studied the ability of the cells with NFATc1 activation to initiate tumorigenesis in nude mice with absence of T cells. We derived tumor cells from NFATc1-induced murine PCa and showed that ~70% of these cells expressed NFATc1 and the HA (human influenza hemagglutinin) tag fused to the C-terminus of NFATc1 (Figure 6a), but no CD45⁺ cells were present (data not shown). These cells were injected to the rear flanks of the nude mice. Tumor growth was detected as early as 4 weeks after the injection in the Dox-treated (with NFATc1 activation), but not in the untreated (without NFATc1

activation), recipient mice (Figures 6b-d). To further test the dependency of tumor growth and progression on NFATc1 activation, we stopped Dox treatment in a subgroup of these mice. Existing tumors started to shrink within days after Dox withdrawal (Figure 6d). This trend was reversed when NFATc1 activation was restored with Dox treatment (Figure 6d), indicating a continuous dependency of the PCa on NFATc1 activation, similar to that seen in cases of oncogene addiction.²⁹⁻³¹ Histopathological analyses of tumors revealed that these allografts contained carcinoma with a more solid growth pattern but showed cytological features similar to those seen in original tumors (Figure 6e), including the presence of a large number of NFATc1⁺/E-Cad⁺ cells (Figure 6f) and STAT3 activation in both NFATc1⁺ and NFATc1⁻ cells that intermingled within the tumor

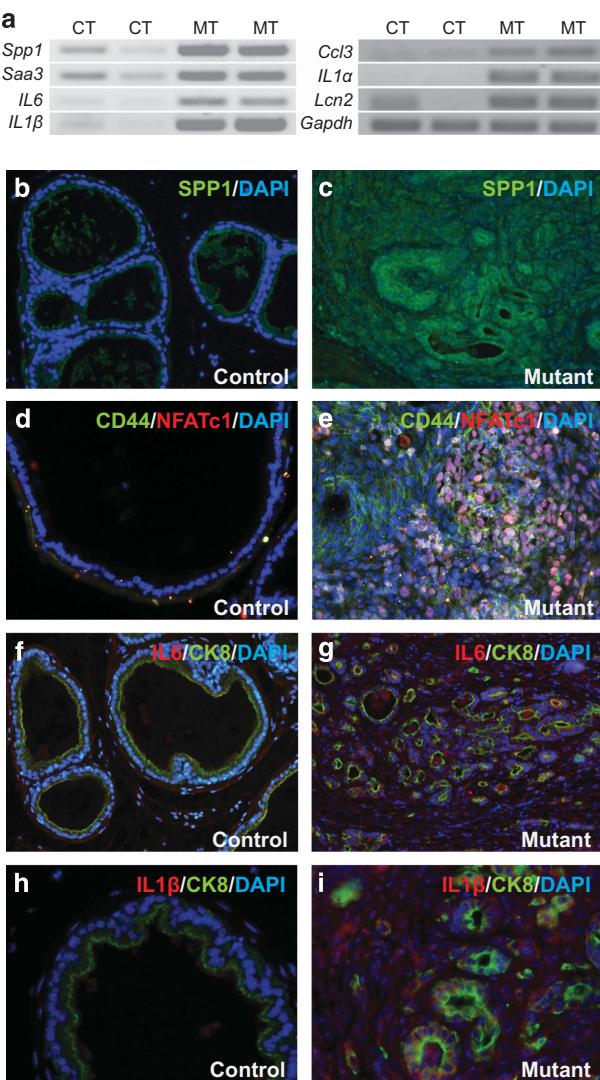


Figure 5. Increased expression of a number of secreted factors in NFATc1-induced PCa. **(a)** Number of secretory factors known to play in PCa progression like SPP1, IL6, IL1 β , IL1 α , along with several other genes were evaluated for transcriptional changes with RT-PCR using RNA from prostates of control (CT) and mutant (MT) mice treated with Dox for 14 weeks. **(b–i)** Immunostaining showed more extensive expression of Spp1, CD44, IL6, and IL1 β in PCa from the prostates of mutant mice compared to their littermate controls.

proper (Figure 6g). As inflammatory cytokines, such as IL6, are similarly upregulated in the grafts (Figure 6h), the establishment of the inflammatory tumorigenic microenvironment thus appears independent of T cells, but likely dependent on the tumor cells, local tissue resident cells, and other immune cells in this model.

NFAT signaling can overcome androgen deprivation to drive PCa progression

Androgens are critical both for development and function of the prostate gland and for the survival and proliferation of the epithelial cells.³² To determine whether NFATc1-induced PCa would respond to hormone deprivation therapy, we analyzed prostates from 18-week-old mutant mice with NFATc1 activation, as weaning and were either castrated or mock-castrated at 14 weeks of age. PCa samples from castrated and mock-castrated mutants are similar in tumor size and histopathological features (Figures 7a and b). However, the distinct nuclear AR staining seen

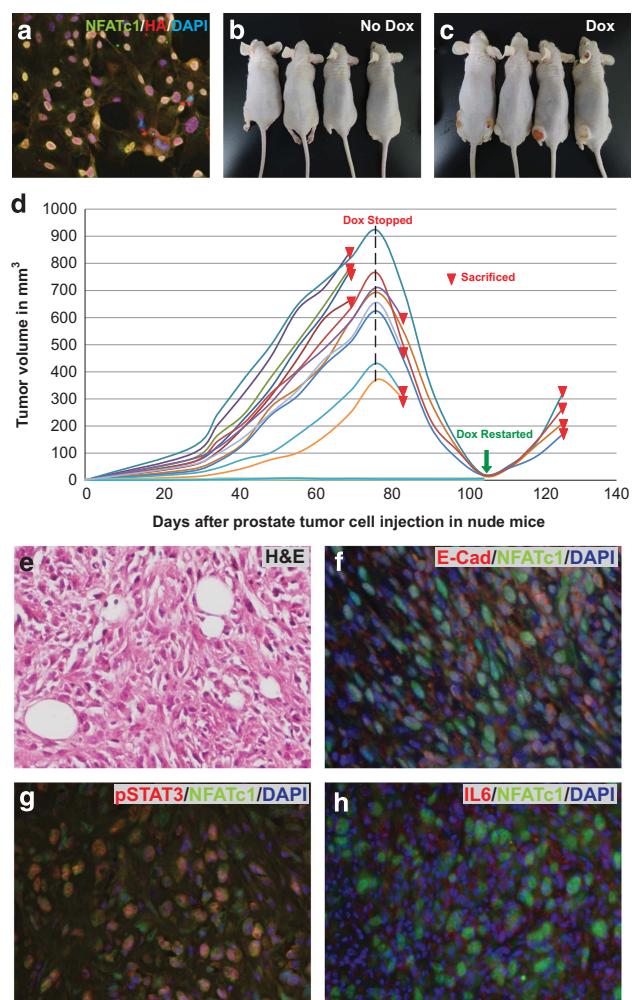


Figure 6. Allografts of NFATc1-induced tumors showed continuous dependency on NFATc1 for tumor progression and survival but not on T-cell functions. Cells from NFATc1-induced PCa samples were isolated and cultured. Most of the cultured cells expressed NFATc1 and the HA tag. **(a)** Cultured tumor cells were injected subcutaneously into the lower flanks of nude mice. 100% of the Dox-treated recipients developed tumors by 4 weeks, whereas none of the untreated mice did. **(b–d)** Termination of Dox treatment resulted in significant decrease in tumor size. Such decrease was reverted if Dox treatment was restarted. **(d)** Representative images of H&E-stained nude mice allograft. **(e)** The allograft tumors predominantly consist of NFATc1⁺ cells expressing E-Cad. **(f)** Similar to the original tumor, extensive pSTAT3 (g) and IL6 (h) expression was observed in the allograft tumors.

in the prostates of non-castrated mice was replaced by a more diffused and weaker expression pattern in castrated mice, indicating that castration had effectively reduced AR signaling in prostatic cells (Figures 7c and d). Tumors from both mock-castrated and castrated mice had significant number of PCNA⁺ proliferating cells, indicating that androgen deprivation had little or no effect on NFATc1-induced PCa and their proliferation (Figures 7e and f).

NFATc1 activation synergizes with the PI3K-AKT pathway to promote PCa progression

Pten is one of the most frequently mutated tumor suppressors in PCa.^{33,34} To understand whether and how the NFAT and PI3K-AKT pathways interact in PCa, we generated mice with both Pten

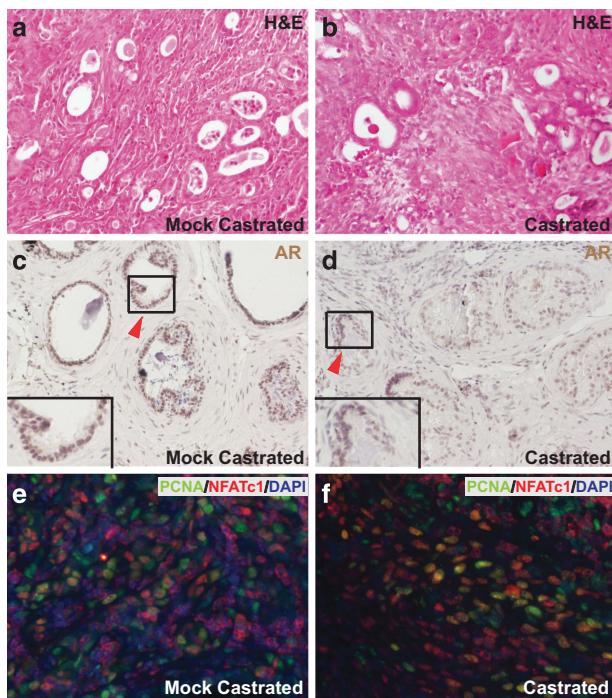


Figure 7. NFATc1-induced tumors are castration-resistant. Representative images of H&E-stained sections of tumors from mock-castrated and castrated mice showing PCa. (**a, b**) Predominantly nuclear AR is present in mock-castrated mice. AR signal is weak and diffuse in the sample from the castrated mutants. The insets represent higher magnification images of the black rectangles indicated by arrows. (**c, d**) The numbers of proliferating cells in mock-castrated and castrated mice expressing NFATc1 and PCNA markers (**e, f**) are very similar.

deficiency and NFATc1 activation in prostatic epithelia. At 10 weeks of age, most *PCre/+;Pten^{fl/fl}* mice with only PTEN deficiency in the prostate epithelium showed enlarged anterior prostates, whereas control and *PCre/+;RT/+;TN/+* mice with only NFATc1 activation starting from P21 in prostatic epithelium had no visible tumors. Interestingly, all double mutants (*PCre/+;RT/+;TN/+;Pten^{fl/fl}*) with both PTEN deficiency and NFATc1 activation developed significantly larger tumors in all prostate lobes when compared with mice of the same age with either *Pten* deficiency or NFATc1 activation alone (Figures 8a–d). The average prostate weight in double mutants (6026.24 ± 1946.85 mg) was increased 17.41-fold when compared with the controls (346.85 ± 36.66 mg), 15.45-fold when compared with mice with NFAT activation alone (390.28 ± 73.16 mg), 7.35-fold when compared with *Pten* null mice (819.14 ± 139.4 mg, Figure 8q). Histopathological analyses revealed that *Pten* null mice and mice with NFATc1 activation alone had PIN at this time, whereas double mutants already had poorly differentiated prostatic adenocarcinoma (Figures 8e–h). Although levels of pAKT were low in prostates from controls and mice with only NFATc1 activation, increased expression of pAKT was apparent in *PCre/+;Pten^{fl/fl}* and *PCre/+;RT/+;TN/+;Pten^{fl/fl}* samples, indicating that the PI3K-AKT pathway was activated in prostates with PTEN loss (Figures 8i–l). SMA staining revealed intact myofibroblast layers in the prostates from single mutants but widespread disintegration of the SMA layer in double mutants, consistent with invasion of the epithelial cells into the stroma (Figures 8m–p). These findings reveal that NFATc1 activation synergizes with PTEN-AKT pathway for PCa initiation and progression.

NFATc1 activation overcomes PTEN loss-induced cellular senescence through downregulation of cell cycle inhibitors

It has been shown that senescence has a tumor-suppressive role in PTEN-deficient cells, explaining the long tumor latency in murine models with PTEN-deficient prostate.³⁴ The earlier onset and faster progression of PCa in double mutants suggest that NFATc1 activation may allow the tumor cells to avoid the PTEN loss-induced cellular senescence, resulting in accelerated tumor growth. Therefore, we examined markers of proliferation and senescence in prostates from (*PCre/+;Pten^{fl/fl}*), (*PCre/+;RT/+;TN/+*), and (*PCre/+;RT/+;TN/+; Pten^{fl/fl}*) mice. The *PCre/+;RT/+;TN/+; Pten^{fl/fl}* tumors had significantly higher levels of proliferation ($68.87 \pm 18.37\%$) than the *PCre/+;Pten^{fl/fl}* ($24.16 \pm 6.76\%$), and *PCre/+;RT/+;TN/+* ($33.46 \pm 3.72\%$) tumors, as assessed by PCNA staining (Figures 9a–d, m). Furthermore, there was a marked decrease in the expression of the senescence marker p21 in *PCre/+;RT/+;TN/+;Pten^{fl/fl}* samples when compared with the *PCre/+;Pten^{fl/fl}* mice (Figures 9e–h, arrowhead and inset). p21 staining was predominantly nuclear in *PCre/+;Pten^{fl/fl}* prostates ($63.6 \pm 7.95\%$). In contrast, nuclear p21 expression was absent in *PCre/+;RT/+;TN/+;Pten^{fl/fl}* ($4.2 \pm 1.30\%$) prostates, where cytoplasmic p21 was occasionally observed (Figure 9n). Although nuclear p21 is considered as tumor suppressors, cytoplasmic p21 may have antiapoptotic roles and enhance cell survival.^{35,36} To further confirm that NFATc1 activation overcomes PTEN loss-induced cellular senescence, we stained for senescence-associated β -galactosidase (SA- β -gal) activity in the prostates. Control and *PCre/+;RT/+;TN/+* prostates showed very few senescent cells, 1% and $6.66 \pm 0.5\%$, respectively. In contrast, $65.6 \pm 8.7\%$ cells within the *PCre/+;Pten^{fl/fl}* prostates were SA- β -gal⁺. Such SA- β -gal⁺ cells in the *PCre/+;RT/+;TN/+;Pten^{fl/fl}* prostates were markedly reduced to $5.8 \pm 1.3\%$ (Figures 9i–l, o), supporting the hypotheses that NFATc1 overcomes Pten-induced cellular senescence by downregulating cell cycle inhibitors.

DISCUSSION

In this study, we have shown higher levels of NFATc1 expression in human PCa specimens and in PCa cell lines when compared with non-neoplastic prostates and non-tumorigenic prostatic cells (Figure 1), consistent with a recent reports of NFATc1 expression in human PCa specimens.⁷ By using a mouse model for prostate-specific and Dox-inducible NFATc1 activation, we have demonstrated that NFATc1 activation in luminal prostatic epithelial cells causes prostate adenocarcinoma with histopathological features similar to the most common type of human prostate adenocarcinoma (Figures 2 and 3). We further showed that NFATc1 activation promotes PCa by upregulating key oncogenic proteins and by establishing a promitogenic and proinflammatory microenvironment (Figures 4 and 5). We have further demonstrated that NFATc1 activation can overcome PTEN loss-induced cellular senescence, greatly accelerate PCa progression associated with PTEN deficiency (Figures 8 and 9). Our results from the mouse model provide the direct *in vivo* evidence that NFATc1 can function as a robust oncogene in prostate tumorigenesis.

The upregulation of c-MYC and activation of pSTAT3 in both NFATc1⁺ and NFATc1⁻ cells cannot be the sole direct results of transcriptional regulation by NFATc1. Instead, in addition to the apparent cell autonomous effects of NFATc1 activation in the regulation of c-MYC, part of the impact from NFATc1 activation appears to go through the production of one or more secreted factors whose existence was first suggested by Neal *et al.*⁴ when studying the effects of NFATc1 activation in cultured 3T3-L1 cells. We have demonstrated the increased expression of multiple cytokines (including IL6, IL1, SPP1 and so on, Figure 5) that have the potential to initiate, or at least contribute to, a proinflammatory and promitogenic microenvironment. Multiple recent studies

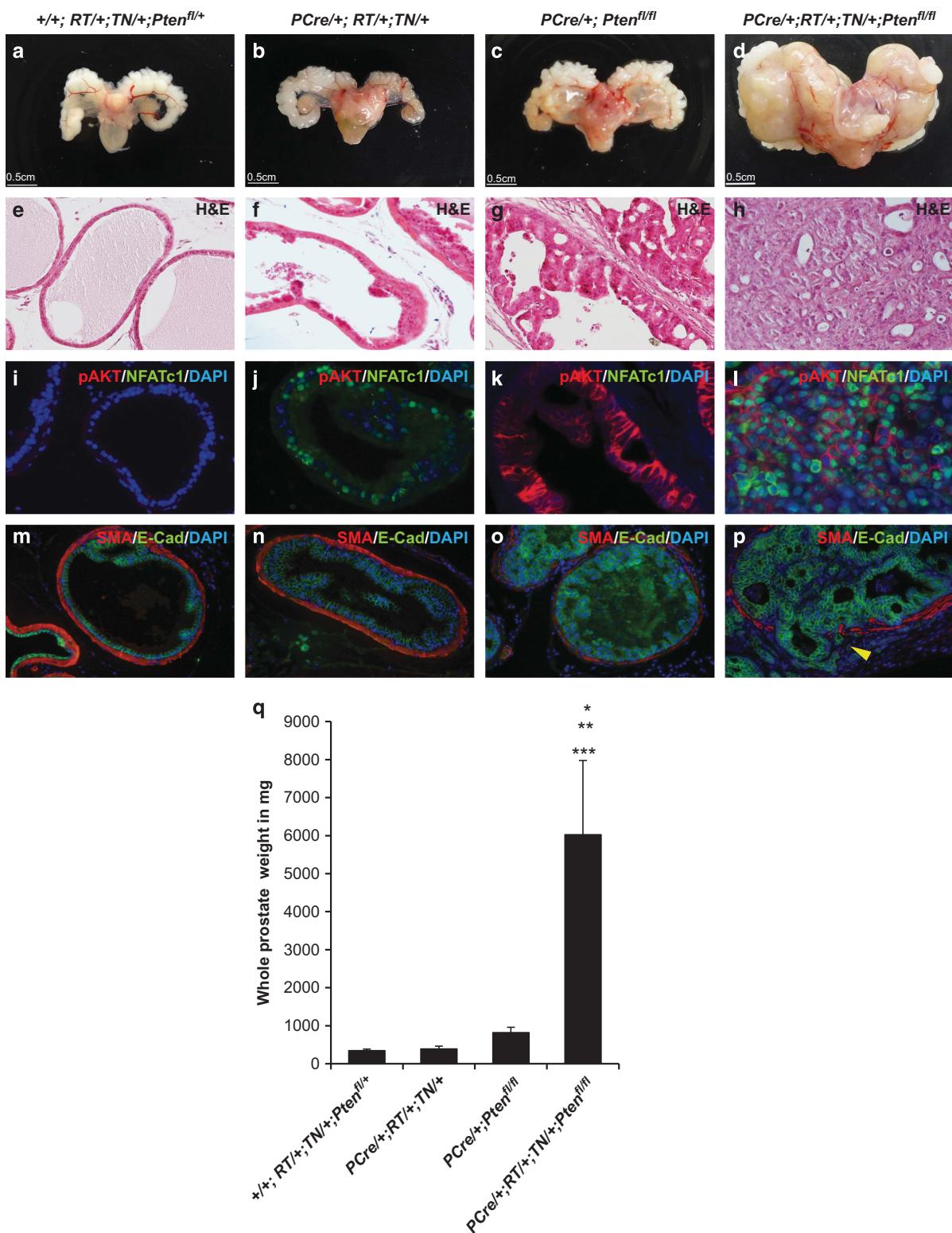


Figure 8. NFATc1 and PI3K-Akt signaling pathway synergize to drive accelerated tumor formation. Representative images of tumors with *PCre/+;RT+/+;TN+/+;Pten^{f/f}* double mutants showing significantly enlarged tumors compared with control (no NFATc1 activation or *Pten* deletion), *PCre/+;RT+/+;TN+/* (NFATc1 activation alone), and *PCre/+;Pten^{f/f}* (*Pten* deletion alone) groups. (a–d) H&E staining of prostates at 10 weeks of age reveals normal glands in controls, PIN in *PCre/+;RT+/+;TN+/* mice, morphologically more advanced PIN in *PCre/+;Pten^{f/f}* mice, and advanced PCa in *PCre/+;RT+/+;TN+/+;Pten^{f/f}* double mutant mice. (e–h) Deletion of *Pten* results in activation of AKT in *PCre/+;Pten^{f/f}* and *PCre/+;RT+/+;TN+/+;Pten^{f/f}* mutant mice, whereas no significant levels of pAKT were detected in control and NFATc1 activation only groups. (i–l) Discontinuation of the SMA⁺ fibromuscular layer and invasion of the E-Cad⁺ cells into the stroma (arrowhead in p) can be seen in the mutants. (m–p) Average whole-prostate weight of the *PCre/+;RT+/+;TN+/+;Pten^{f/f}* mice is drastically higher than those of the +/+;RT+/+;TN+/+;Pten^{f/f} mice (*P < 0.05, n = 7) *PCre/+;RT+/+;TN+/* mice (**P < 0.05, n = 7) and *PCre/+;Pten^{f/f}* mice (**P < 0.05, n = 7). (q) All data are presented as mean ± s.d. Two-tailed t-tests were performed for comparison between groups.

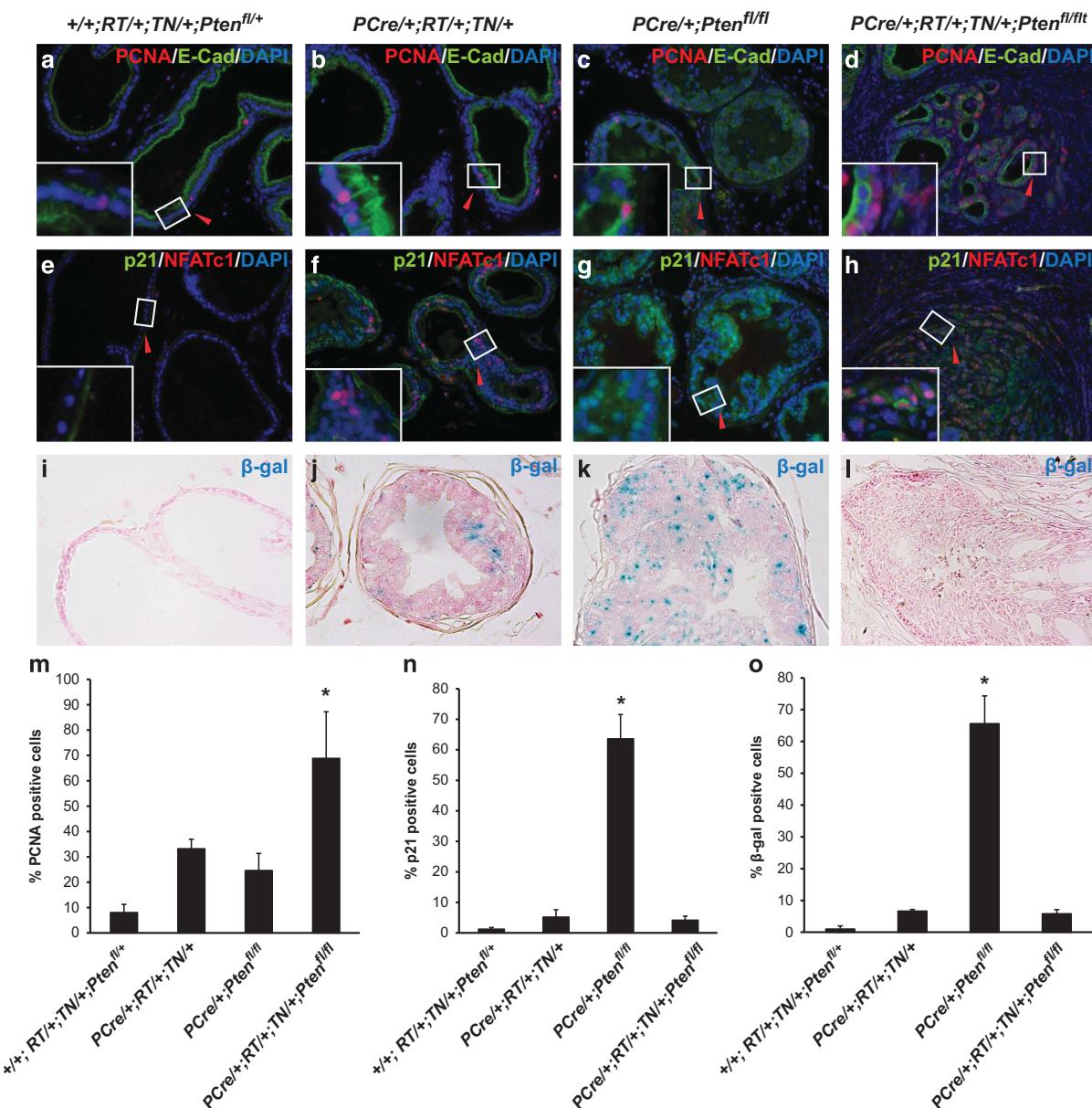


Figure 9. NFAT Activation overcomes PTEN loss-induced cellular senescence in PCa. $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/+; \text{Pten}^{fl/fl}$ mutant prostates have a much larger number of proliferating E-Cad⁺ cells when compared with control, $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/$, and $\text{PCre}^+/\text{Pten}^{fl/fl}$ mice. (a–d) The insets are higher magnification images of the white rectangles indicated by arrows. Quantification of PCNA staining of 10-week-old prostates is shown in m. Asterisk indicates statistical significance between $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/+; \text{Pten}^{fl/fl}$ double mutants and $\text{PCre}^+/\text{Pten}^{fl/fl}$ single mutants (* $P < 0.05$, n=5). (e–l) Senescence analysis of prostates through p21 and SA- β -gal staining. Control and $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/$ mutant lack p21-expressing cells. Mostly nuclear p21 expression was seen in large numbers of prostate epithelial cells in the $\text{PCre}^+/\text{Pten}^{fl/fl}$ mice, whereas such cells are essentially absent in the $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/+; \text{Pten}^{fl/fl}$ double mutants (* $P < 0.05$, n=5) (e–h, n). The insets represent higher magnification images of the white rectangles indicated by arrows. Quantification of p21⁺ cells in 10-week-old prostates is shown in n. (i–l) Senescence-associated β -gal staining of prostates from 10-week-old mice. Prostates from control and $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/$ mice had very few senescent cells. Prostates of the $\text{PCre}^+/\text{Pten}^{fl/fl}$ mice contain a large number of senescent cells, when compared with prostates of the $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/+; \text{Pten}^{fl/fl}$ double mutants showing drastically fewer p21⁺ senescent cells (* $P < 0.05$, n=5) (i–l, o). Quantification of SA- β -gal⁺ cells in 10-week-old prostates (o) is consistent with the p21 data. Prostates from control and $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/$ mice had few SA- β -gal⁺ senescent cells. Prostates of the $\text{PCre}^+/\text{Pten}^{fl/fl}$ mice contain a large number of SA- β -gal⁺ senescent cells, when compared with prostates of the $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/+; \text{Pten}^{fl/fl}$ double mutants that had significantly fewer SA- β -gal⁺ senescent cells (* $P < 0.05$, n=5). All data are presented as mean \pm s.d. Two-tailed t-tests were performed for comparison between groups. Asterisk indicates statistical significance between the $\text{PCre}^+/\text{Pten}^{fl/fl}$ single mutants and the $\text{PCre}^+/\text{RT}^+/+; \text{TN}^+/+; \text{Pten}^{fl/fl}$ double mutants.

have collectively indicated that SPP1 is one of the four key signatures genes correlated with PCa progression and prognosis.²⁷ Direct transcriptional regulation of SPP1 by NFAT has been reported in arteries,²⁸ consistent with our finding of increased SPP1 levels in mice with NFATc1 activation. Elevated IL6 levels are

found in human PCa specimens and even in serum of patients with untreated metastatic or castration-resistant prostate cancer where IL6 levels correlate negatively with tumor survival and response to chemotherapy.^{37,38} NFATc1 activation-induced upregulation of IL6 and other proinflammatory cytokines

(Figures 4 and 6) can activate the JAK-STAT pathway in both NFATc1⁺ and NFATc1⁻ cells, as revealed by our data of STAT3 activation in the PCa (Figure 4), leading to promitogenic effects, including the upregulation of c-MYC that is known to be a target of STAT3 regulation.^{39–41} Clinical trials with anti-IL6 antibody therapy has not yet demonstrated beneficial effects in advanced PCa.^{37,38} Although there may be many explanations, the wide range of cytokine activation in the microenvironment, as seen in the NFATc1-induced PCa, predicts that monotherapy targeting any one of them would not be particularly effective.

As our data support the notion that NFATc1 can be a potent oncogene for PCa through its cell autonomous and non-autonomous effects, we went a step further to ask if and how NFATc1 activation may interact with other commonly occurring PCa mutations. PTEN is an important tumor suppressor and is frequently mutated in PCa.⁴² By introducing NFATc1 activation to mice deficient for *Pten*, we have showed synergistic effects of NFATc1 activation and *Pten* inactivation/Akt activation, as evident by the earlier onset and greater aggressiveness of the PCa in the double mutants. The mechanistic base of such synergism is likely that the introduction of the NFATc1 activation confers the cells the ability to overcome PTEN loss-induced cellular senescence (Figures 8 and 9). Although the detailed mechanism for the anti-senescence effects of NFATc1 activation requires further investigation, it is possible that NFATc1-Interleukins-JAK-STAT3 axis upregulates the expression of SKP2, which in turn, suppresses the expression of p21. This hypothesis is consistent with the observations that SKP2 can be induced by activated STAT3 and that SKP2 negatively regulates p21.^{43,44} Regardless of the specific pathways affected, the anti-senescence effects of NFATc1 activation can conceivably enhance the tumorigenic effects of mutations in other genes associated with prostate tumorigenesis.

We have so far not been able to find evidence of metastasis of the NFATc1-induced PCa in mice with NFATc1 activation in the prostate for as long as 5 months. Although it is possible that metastasis may occur if the PCa progresses further, this cannot be determined in the current system we use due to lesions in the ears of the mutant mice that result in the euthanasia of the mice by animal study protocol. The exact cause of these lesions is unknown but appears to be related to ectopic expression of the *PCre* transgene used. As such, the question of potential metastasis in older mice will be investigated when more specific Cre drivers become available.

Although we have shown that NFATc1 activation drives PCa initiation and progression, we do not expect that genetic mutations of the NFATc1 gene to be a major mechanism for NFATc1 activation. This is because that the ligand-independent activation of NFATc1 requires blocking multiple phosphorylation sites. Random mutations within NFATc1 will most likely inactivate but not activate the protein.³ Instead, the activation of NFATc1 is likely a result of mutations in genes whose protein products act upstream of NFATc1. One of such upstream factors may be TRPV6 that has been linked to human PCa and thought to enhance proliferation and apoptotic resistance through the upregulation of the calcium-calcineurin-NFAT pathway.⁶ As NFATc1 activation in the prostate has pleiotropic effects on the cells expressing it and on the neighboring cells through alterations in multiple signaling factors in the microenvironment, inhibiting NFATc1 activation could be more effective than targeting one or more of the downstream pathways and factors in treating cancers with NFATc1 activation. The recent findings that calcineurin-NFAT inhibitors suppressed proliferation, migration, and invasion of cultured PCa cells,⁷ as well as that Silibinin suppressed the PCa cells-induced osteoclastogenesis partially through the inhibition of NFATc1¹⁰ are encouraging and in broad agreement with data presented in this report. Our *in vivo* data on oncogene addiction and the ability of NFATc1 activation to continue to drive PCa progression after castration further suggest an oncogenic role of

NFAT in castration-resistant prostate cancer and the potential benefits in inhibiting NFAT pathway in fighting PCa.

MATERIALS AND METHODS

Mouse (*Mus musculus*) strains and Dox treatment

All animal studies were approved by the Washington University Animal Studies Committee and have been conducted according to relevant NIH guidelines. The *PCre4-Cre* (*PCre*+/), *ROSA*^{ERT1}(*RT*), *Teto-NFATc1*^{Nuc} (*TN*) strains and the genotyping methods were described previously.^{17,21–23} For *NFATc1*^{Nuc}, the substitution of the serines targeted for phosphorylation and dephosphorylation with alanines renders the modified NFATc1 proteins constitutively nuclear and active. For studying interactions between NFAT and PTEN, *PCre* mice were crossed with *Pten*^{fl/fl} mice (The Jackson Laboratory, Bar Harbor, ME, USA) to generate *PCre*/+; *Pten*^{fl/+} males. These *PCre*/+; *Pten*^{fl/+} mice were then crossed to *RT/RT*; *TN*/+; *Pten*^{fl/fl} mice to generate controls and *PCre*/+; *RT*/+; *TN*/+; *Pten*^{fl/fl} mutants. Dox was given through drinking water provided *ad libitum* at 2 mg/ml, starting at P21. Drastic morphological differences makes blinding of specimens from different genotype groups ineffective in this study.

Statistical analyses

We use all available specimens that meet the quality standards. All quantitative data are presented as mean \pm s.d. Two-tailed *t*-tests were performed between groups and *P* < 0.05 is considered significant.

Human prostate specimens

Formalin-fixed paraffin-embedded human specimens were obtained from the archives of prostate biopsies in the Department of Pathology and Immunology at Washington University School of Medicine. The human studies protocol was approved by the IRB at Washington University School of Medicine. Tissue Microarrays containing human non-neoplastic prostate specimens and prostatic adenocarcinomas with Gleason scores 5–9 were obtained from Biomax.

Histology, immunostaining and western blotting

Mouse tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 7 μ m were collected and stained with Hematoxylin and Eosin. Immunostaining was performed as described.²⁵ In brief, deparaffinized sections were hydrated and antigen retrieval was done using citrate buffer (10 mM, pH6), using a steamer. These slides were washed with 100 mM glycine for 2 \times 10' and incubated with primary antibodies in humidified chamber overnight at 4 °C. After washing with phosphate-buffered saline with tween 3 \times 5', samples were incubated with secondary antibodies for 1 h at room temperature and washed with phosphate-buffered saline with tween 4 \times 5'. After incubation with 4',6-diamidino-2-phenylindole for 5', the slides were washed again and mounted with Fluoromount G for imaging (Southern Biotech, Birmingham, AL, USA). Primary antibodies used were: from Abcam, Cambridge, UK: rabbit polyclonal Anti-ECadherin (ab53033 1:100), rabbit polyclonal anti-CK5 (ab24647, 1:100), mouse monoclonal anti-p63 (ab53039, 1:100), rabbit polyclonal anti-androgen receptor (ab47570, 1:100), rabbit polyclonal anti-SPP1 (ab8448, 1:100), mouse monoclonal anti-synaptophysin (ab18008, 1:100), rabbit polyclonal anti-IL6 (ab6672, 1:200), rabbit polyclonal anti-c-MYC (ab39688, 1:100); from Life Technologies, Carlsbad, CA, USA: rat monoclonal anti-CD44 (558739, 1:100), mouse monoclonal anti-NFATc1 (556602, 1:100), Alexa Fluor 488 and 555-conjugated secondary antibodies (1:1000); from Cell Signaling Technologies, Danvers, MA, USA: rabbit polyclonal anti-Phospho STAT3 (9131, 1:100), rabbit monoclonal anti-STAT3 (4904, 1:50), rabbit monoclonal anti-pAKT (4060 s, 1:100); from Sigma, St Louis, MO, USA: mouse monoclonal anti-qSMA (A2547, 1:100), horseradish peroxidase-conjugated secondary antibodies (1:1000); from Santa Cruz Technologies, Santa Cruz, CA, USA: rabbit polyclonal anti-IL1 β (sc7884, 1:100), rabbit polyclonal anti-p21 (sc-471, 1:50); from Developmental Studies Hybridoma Bank, Iowa City, IA, USA: rat monoclonal anti-Cytokeratin 8 (TROMA-1, 1:100); from Bethyl Labs, Montgomery, TX, USA: rabbit monoclonal anti-PCNA (IHC-00012, 1:100).

For western blotting, protein extracts were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and probed by appropriate primary antibodies. After incubation with Alexa 680-conjugated goat anti-rabbit IgG (Life Technologies) and IRDye 800CW-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA, USA)

secondary antibodies, antibody complexes were visualized by an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

RT-PCR

Total RNA was isolated using Trizol reagent (Life Technologies) and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was prepared by using the ThermoScrip RT-PCR System (Life Technologies). PCR conditions were: 95 °C, 4', 35 × (94 °C 35'; 55–59 °C, 35'; 72 °C, 35'). Primers used for 59 °C annealing were: NFATc1-Fwd AAGAAGATGGTCCTG TCTGG and NFATc1-Rev GTAGTCTGGTACGTGTC; IL6-Fwd TGTGCAAT GGCAATTCTGAT and IL6-Rev-GGTACTCCAGAACGAGAGGA; IL1β-Fwd CTGTGGCAGCTACCTGTGTC and ILβ-Rev TAATGGGAACGTACACACC. Primers used for 58 °C annealing were: Ccl3-Fwd CTGCCTGCTGCTTCT CCTAC and Ccl3-Rev CCCAGGTCTTTGGA-GTCA; IL1α-Fwd CAGTCTGCCA TTGACCATCT and IL1α-Rev CTCCCTGAAGGTGAAGTTGGA; Lcn2-Fwd AAACAGAAAGGCAGCTTACGA and Lcn2-Rev CCTGGAGCTTGGAAACAAATG; Gapdh-Fwd CACTCTCACCTCGATG and Gapdh-Rev TGCTGTAGC CGTATTCTATTG. Primers used for 56 °C annealing were: Saa3-F CCGTGAACCTTGAAACAGCCT and Saa3-R TGCCATCATTCTTGATCTGA; Primers used for 55 °C annealing were: Spp1-Fwd TGGTGCTGACC CATCTCA and Spp1-Rev GTTTCTGCTTAAAGTCATCCTTTCTT.

Cell culture

For primary tumor cell culture, prostates from *PCre/+;RT/+;TetO-NFATc1^{Nuc}* mice, treated for 14 week since P21, were harvested and cut into cubes <1 mm³ and cultured in Dulbecco's modified eagle medium-F12 (10% fetal bovine serum, 5% penicillin/streptomycin and 2 µg/ml Dox). Cells grew out of the tumor chunks were fed with fresh media every 2–3 days. A subculture with predominantly epithelial cells was established and expanded for subsequent experiments. RWPE-1, PC3, LNCaP and DU145 cells were originally obtained from the American Tissue Culture Collection (ATCC) and maintained according to ATCC guidelines. PC3, LNCaP and DU145 cells were grown in Rosewell Park Memorial Institute media supplemented with 10% fetal bovine serum. RWPE-1 cells were maintained in keratinocyte-serum-free media supplemented with epidermal growth factor and bovine pituitary extract.

Tumor graft in nude mice

For allografts, 3 × 10⁶ aforementioned cultured tumor cells were injected subcutaneously into lower flanks of 16 male NCr nude mice (6–8-week-old, Taconic, Hudson, NY, USA). These mice were randomized into four groups. Group 1 was untreated and groups 2–4 were Dox-treated right after tumor cell injection. Group 2 was killed on the 70th day. Dox was stopped for group 3 on the 77th day and the mice were killed on the 84th day as tumors started to recede. Dox was stopped for group 4 on the 84th day and restarted on the 105th day. Group 4 was killed on the 126th day. Tumor volume was determined using a previously described formula.⁴⁵

Castration

After anesthesia with 80 mg/kg ketamine and 5 mg/kg xylazine, each testis was gently pushed into the scrotum and surgically removed through a 0.5 cm incision. The spermatic cord and vascular plexus were tied with sterile suture. Wound clips were used to close the incision and were removed 1 week after surgery.

Senescence assay

Tissues sections were assessed for senescence by staining for SA-β-gal activity.⁴⁶ In brief, prostate cryosections were fixed at room temperature for 3 min in 0.2% glutaraldehyde and 2% formaldehyde, rinsed with phosphate-buffered saline and incubated overnight at 37 °C in SA-β-gal staining solution (40 mM citric acid, 40 mM H₂NaPO₄·2H₂O, 5 mM K₄Fe(CN)₆·3H₂O, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 150 mM NaCl, pH 6.0), containing 1 mg/ml X-gal (US Biochemical, Cleveland, OH, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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